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14. ABSTRACT

The goal of this project is to evaluate the effect of a moderate-level brain injury on risk for opioid abuse using preclinical models of abuse-related behaviors in rats. Thus far we have assessed the effect of brain injury on the rewarding effects of oxycodone in two rat self-administration procedures. We have found that there are significant differences in the acquisition and maintenance of oxycodone intravenous self-administration behavior between brain-injured and control rats. Data collected to date suggest brain injured rats have a greater sensitivity to the rewarding effects of oxycodone and a greater tolerance for the use-limiting effects of oxycodone (eg. sedation, motor impairment, dysphoria). Conversely, it appears that there is no difference between brain-injured and sham controls in a model of relapse to oxycodone self-administration. Preliminary testing of oxycodone for analgesic strength and development of tolerance also has shown no difference between sham controls and brain injured subjects. Additional self-administration studies to determine the rewarding strength of oxycodone as well as development of physical dependence are ongoing.

15. SUBJECT TERMS
Traumatic brain injury, drug abuse, oxycodone, opioid, preclinical models, rat

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Progress Report for DoD Peer Reviewed Medical Research Program of the Office of the Congressionally Directed Medical Research Program FY10 Investigator-Initiated Research Award: Partnering PI Option Application entitled "Opioid Abuse after TBI"

Prepared by Katherine L. Nicholson, DVM, Ph.D.

July 2013

Introduction:

Data from both military and civilian studies indicate that drug abuse rates are increased by the occurrence of a traumatic brain injury (TBI), but the underlying reasons for this remain unclear. This relationship between TBI and drug abuse is particularly alarming given the significant numbers of military personnel who experience a brain injury and the growing numbers of persons who are prescribed opioid pain medications. There is significant overlap in anatomical brain regions involved in reward pathways associated with addiction and the brain regions commonly damaged in TBI which suggests that TBI could alter the reward circuitry, thereby increasing the likelihood of opioid abuse and addiction. Given the overlap in affected brain regions and growth factors, we speculated that TBI could result in neurological changes that increase vulnerability for drug abuse and addiction. Consequently, we have been evaluating the effects of TBI on both the rewarding effects of opioid drugs as well as the development of tolerance in well-established rat models of abuse-related drug effects. We are using lateral fluid percussion injury to induce a moderate level of injury in adult, male Sprague-Dawley rats. Our opioid compound selected for study is one of the most commonly prescribed and also misused/abused prescription pain medications, oxycodone. To assess the rewarding effects of oxycodone after TBI, we have used intravenous self-administration procedures to evaluate the acquisition and maintenance of oxycodone self-administration as well as the risk of relapse to oxycodone self-administration following extinction of the behavior. We have found that there are significant differences in the acquisition and maintenance self-administration behavior between brain-injured and control rats. Data collected to date suggest brain injured rats have a greater sensitivity to the rewarding effects of oxycodone and a greater tolerance for the use-limiting effects of oxycodone (eg. sedation, motor impairment, dysphoria). Conversely, it appears that there is no difference between brain-injured and sham controls in a model of relapse to oxycodone self-administration. We are also investigating the acute analgesic effects of oxycodone after TBI and the effect of repeated oxycodone administration. Preliminary testing for analgesic strength and development of tolerance has shown no difference between sham controls and brain injured subjects. Additional self-administration studies to determine the rewarding strength of oxycodone as well as development of physical dependence are ongoing.

Body:

Our progress in completion of the aims is detailed below. A brief description of the aims, the work conducted, and the data collected respective to the statement of work is provided after the original text of the aims and statement of work (modification requested and approved June 21, 2013), which are denoted by bolded and italicized font, respectively.

Aim 1: Test the hypothesis that mild or moderate TBI in adolescent or adult rats, causes changes in reward circuitry and dopamine signaling as indicated by enhanced methamphetamine-induced locomotor sensitization.

Aim 2: Investigate the hypothesis that mild or moderate TBI increases the susceptibility for psychomotor-stimulant abuse as measured by an alteration in the reinforcing properties of methamphetamine in a self-administration paradigm.

Aim 3: Examine the hypothesis that mild or moderate TBI in rats induces structural changes in brain regions associated with reward/risk circuitry including the nucleus accumbens, amygdala, hippocampus, and prefrontal-parietal white matter tracts.

Update on Tasks from Year 2 Statement of Work:

General Study Overview:

This project evaluating the impact of traumatic brain injury on the risk of opioid drug abuse will be completed in 3 years. Given the labor-intensive nature of certain components of the study, work on multiple specific aims will occur concurrently to utilize resources most efficiently.

- *Dr. Floyd will travel to VCU and perform the injury procedures over 2 to 3 day periods on five occasions during the first year and on 3 occasions during subsequent years.*

Dr. Floyd made 4 trips to VCU to perform injury procedures in the first year. The 5th trip was cancelled due to the fact that she broke the scaphoid bone in her hand in a fall wherein she landed on her outstretched hand. Because of the injury she was unable to perform the injury procedure for several months and could only perform a limited number of surgeries during each of two visits in Fall 2012 due to limited mobility/persistent pain. She therefore made 4 additional trips to VCU during Spring/Summer of 2013 to compensate.

- *In the first year visits, Dr. Floyd will conduct the TBI procedure and train personnel at VCU to perform the procedure in order to permit larger numbers of animals to be prepared during years 2 and 3 as detailed below.*

Dr. Floyd conducted all the TBI procedures the first year and the majority in the second year. She has trained Dr. Nicholson and her student in performance of the craniectomy procedure. Dr. Nicholson is reliably performing the craniectomies on her own and inducing injury under the supervision of Dr. Floyd to ensure consistency.

- *During the first two years, we will generate TBI subjects for studies to evaluate tolerance production using two pain models as well as acquisition of oxycodone self-administration.*

The team has these tasks well underway and the data collection is nearing completion for these studies. The details of the animals conducted thus far are listed below.

- *Testing of subjects in other aspects of self-administration behavior (reinstatement and reinforcing efficacy) and development of physical dependence will begin late in year two and extend into year three.*

Testing of subjects in the reinstatement procedure was initiated and will be completed early in Y3. The physical dependence study was deferred to year three in the SOW modification in order to redistribute costs to accommodate added subjects in the self-administration study.

- *For the tolerance and dependence procedures, the goal is to generate 10 subjects per treatment condition as outlined in the research design for a total of 80 test subjects completing evaluation of the antinociceptive effects of oxycodone following acute and chronic administration and 40 subjects completing assessment of development of physical dependence. The 10 subjects completing each treatment condition will be euthanized following the final oxycodone exposure for collection of brains for analysis. The total time required for acclimation, food training (dependence study only), injury and behavioral evaluation of these subjects is approximately 6 to 8 weeks.*

Many animals in the tolerance studies have been included in the experiments thus far and details of the group numbers as well as a summary of the preliminary data are listed below. Both antinociception procedures have been optimized to maximize retention of subjects in the study, a problem encountered during year 1 testing. Additionally implantation of the iPrecio has also been optimized with 90% of pumps being able to be reprogrammed and reimplanted for a second dosing round.

- *Each cohort of self-administration (SA) animals will require 35 to 60 days to complete testing depending on the aspect of SA being assessed. This includes time for acclimation to the laboratory and handling, catheterization surgery and recovery, brain injury and evaluation of acquisition, reinforcing efficacy or reinstatement to oxycodone SA. This time frame is based on exclusive use of 4 self-administration chambers in 2 or 3 runs/day = 8 to 12 animals/day. Therefore, 8 to 12 rats can undergo injury and be*

designated to a SA procedure every 6 to 8 weeks. For all SA studies, the numbers shown below reflect the number of animals entering the different SA paradigms. With an anticipated loss of ~20% of subjects due to premature loss of catheter patency (acquisition) and failure to acquire the baseline behavior (PR and reinstatement procedures) this will result in a total of 10 subjects/treatment group.

Many animals in this cohort have been included in the experiments thus far and details of the group numbers to date as well as a summary of the preliminary data are listed below.

Tasks specific to year two: The animal numbers described are based on a loss of 5% of subjects following sham brain injury and 20% loss following moderate injury.

Year Two *– Continued testing of antinociception and tolerance production with WWTW and hotplate test. Complete testing of acquisition of self-administration of oxycodone across test groups (4 doses: 0.003, 0.01, 0.03 and 0.056 mg/kg/infusion), begin assessing relapse to oxycodone self-administration. Begin assessing acquisition of food maintained behavior and locomotor effects of oxycodone. These rats will be randomly distributed to TBI condition/behavioral procedure and oxycodone dose upon arrival.*

Month	1	2	3	4	5	6	7	8	9	10	11	12
Rats undergoing TBI			22		24				30	28	~~24*~~ 13	53
Rats Surviving TBI			17		22				26	23	~~20*~~ 10	46
Rats→ SA			0		22				26	11	0	26
Rats→TOL			17		0				0	12	10	
Rats → Food/LA												20

* Note: the numbers in the bottom three rows are correct and match the text, numbers of rats undergoing and surviving TBI was incorrect in the revised SOW.

By end of year two:

- *Task 1: 39 additional rats will have completed testing of the antinociceptive effects of oxycodone and the development of tolerance using the WWTW (12 rats) and hotplate test (27 rats) completing all saline controls in both injury conditions as well as 10 subjects under the ED80 repeated dosing condition (50 subjects out of the total 80). These subjects will be euthanized and brains collected for shipment to Dr. Floyd at UAB following the final oxycodone testing.*

- *Task 2: 85 additional subjects will have been entered into evaluation of acquisition of oxycodone self-administration. With a potential loss of 20%, this will provide 10 subjects/treatment condition completing the study (50 subjects total completing acquisition assessment). Once the optimum dose for acquisition has been determined, 19 TBI subjects will be generated to evaluate relapse to oxycodone self administration, with ~14 subjects completing testing.*

- *We will also evaluate sham and brain-injured subjects for acquisition of food maintained behavior. The same subjects will be used for assessing the effects of oxycodone on locomotor activity. Twenty-six subjects will undergo either sham or moderate injury to provide 20 subjects completing food acquisition.*

Subjects for the food maintained behavior study have been purchased and undergone acclimation to facilities and handling but did not enter into the experimental protocol until after VCU IACUC and ACURO approval in July 2013.

1. Overview of milestones completed and in progress

Table 1. Shown is the distribution of animals entered into the study during project Y2 (from 07/01/12 through 06/30/13).

<table>
<tr><td rowspan="8">Total # subjects entered into protocol

=112
(10+10+20+22+24+26)</td><td rowspan="4">Total number catheterized

=62</td><td rowspan="2">Total number undergoing sham injury

=33</td><td>Total entering acquisition
= 23</td><td>Total completing acquisition
= 16</td></tr>
<tr><td>Total entering reinstatement
= 8</td><td>Total completing reinstatement
= 7</td></tr>
<tr><td rowspan="2">Total number undergoing TBI
= 29</td><td>Total entering acquisition
= 13</td><td>Total completing acquisition
= 9</td></tr>
<tr><td>Total entering reinstatement
= 9</td><td>Total completing reinstatement
= 7</td></tr>
<tr><td rowspan="4">Total number implanted with infusion pumps
=50</td><td rowspan="2">Total number undergoing sham injury
=21</td><td>Total number entering WWTW testing
= 6</td><td>Total completing testing/dosing
= 6</td></tr>
<tr><td>Total number entering HP testing
= 14</td><td>Total completing testing/dosing
= 14</td></tr>
<tr><td rowspan="2">Total number undergoing TBI
= 29</td><td>Total number entering WWTW testing
=7</td><td>Total completing testing/dosing
=7</td></tr>
<tr><td>Total number entering HP testing
= 13</td><td>Total completing testing/dosing
= 12</td></tr>
</table>

Animal loss included:

- 18 subjects lost during fluid percussion injury. Two were lost during the craniectomy procedure (considered Sham losses) and 16 subjects were lost due to the injury itself. Of these, 6 subjects were removed from the study due to inadequate levels of injury as we adjusted to the new fluid percussion device at VCU.
- 12 subjects due to loss of catheter patency
- 1 subject due to pump malfunction
- 2 subjects due to failure to acquire self-administration behavior for the reinstatement study

Animal use relative to SOW designated milestones:

General

- Total number of subjects entered into project = 112 (goal 170; includes 54 subjects pending IACUC/ACURO approval)
- Total number surviving head injury procedure = 93 (144 goal; includes predicted 45 surviving subjects allotted to self-administration and food maintained behavior pending IACUC/ACURO approval).

Discrepancy is numbers is due to pending VCU IACUC/ACURO approval for modifications to the SOW granted late in June 2013. An additional 54 animals were purchased in FY2 but did not enter the protocol until after animal use approval in FY3 (July 2013). These 54 subjects have all undergone TBI/sham injury procedures and are actively testing at the time of preparation of this report. All behavioral testing for these animals will be complete by the end of August 2013. Animals slated for testing in July of Y3 have also been purchased, acclimated and 20 have already or will undergo injury and behavioral assessment in July and August 2013 so we remain on track for completion as scheduled in the SOW.

Task 1: 39 additional rats will have completed testing of the antinociceptive effects of oxycodone and the development of tolerance using the WWTW (12 rats) and hotplate test (27 rats) completing all saline controls in both injury conditions as well as 10 subjects under the ED80 repeated dosing condition (50 subjects out of the total 80). These subjects will be euthanized and brains collected for shipment to Dr. Floyd at UAB following the final oxycodone testing.

Thirty-nine tolerance study subjects have undergone injury/sham injury, 38 subjects (12 WWTW, 26 hotplate) have completed antinociceptive testing and repeated saline or oxycodone administration and had brain tissue collected. One subject was removed from the study due to pump failure. The pump is being returned to the manufacturer for replacement.

- Completed testing in WWTW = 15 entered, 13 finished (goal 15 enter, 12 finish). All repeated-saline administration was completed in both sham controls and brain injured subjects. Testing of the ED80 dose of oxycodone for tolerance production has begun – 4 subjects completed.
- Completed testing in hotplate procedure = 35 entered, 26 finished (goal 35 enter, 27 finish). All repeated-saline administration was completed in both sham controls and brain injured subjects. Testing of the ED80 dose of oxycodone for tolerance production has begun – 6 subjects completed, 1 subject removed from analysis due to pump failure mid study.

Task 2: 85 additional subjects will have been entered into evaluation of acquisition of oxycodone self-administration. With a potential loss of 20%, this will provide 10 subjects/treatment condition completing the study (50 subjects total completing acquisition assessment). Once the optimum dose for acquisition has been determined, 19 TBI subjects will be generated to evaluate relapse to oxycodone self administration, with ~14 subjects completing testing.

- Completed testing in self-administration study = 62 entered, 39 completed (goal 85 entered, 59 completed)
- Primary discrepancy in numbers is due to pending IACUC/ACURO approval for modifications to the SOW granted in June 2013. An additional 30 animals were purchased in Y2 but did not enter the protocol until Y3 (July 2013). We also experienced a higher than predicted loss of catheter patency in one cohort. Subjects from the original SOW (acquisition at 0.003, 0.01 and 0.03; reinstatement) have undergone injury and completed all testing. Subjects for the additional acquisition dose of oxycodone (0.056 mg/kg/infusion) have been purchased and undergone acclimation to the facilities and handling but did not enter into the experimental protocol until after VCU IACUC and ACURO approval in July 2013. Twenty-two subjects have been catheterized and undergone injury/sham injury with 14 subjects completing evaluation of relapse-like behavior.

2. Subjects:

Adult male Sprague Dawley rats were purchased from Charles River at age/size ranges predicted to result in body weights of 300-350 g at time of injury. Self-administration and hot plate antinociception subjects were purchased minimally 2 weeks prior to injury permitting time for 7 days of handling and acclimation before intravenous catheterization or pump implantation was performed (see below). Subjects to be used in the warm water tail withdrawal procedure (WWTW) were delivered minimally 3 weeks prior to scheduled injuries in order to fully acclimate them to the restraint tubes.

3. Fluid percussion Injury:

All subjects underwent fluid percussion injury following handling, training and surgical instrumentation for subsequent behavioral procedures. Lateral fluid percussion was induced in adult rats as previously described (Floyd et al., 2002). Anesthesia was induced and maintained with 4% isoflurane. The subjects were surgically prepared and transferred to a stereotaxic device for craneictomy and continued to be maintained under isoflurane anesthesia.

Description of Procedure. An incision (~8mm) was made in scalp and fascia scraped from the skull. A point mid-way between Bregma and Lambda and central suture/lateral ridge was marked on the medial skull surface with sterile tissue marker. A 4.8mm craniotomy was cut with a trephine by hand over the right motor cortex. An injury cannula was fashioned from the

hub of a female leur-lock 20g needle by affixing the plastic tube to the skull with glue and securing with dental acrylic. After the acrylic hardened (15 minutes), the injury cannula was filled with sterile saline, and the brain injury induced by compressing the sterile saline with the fluid percussion device(Custom Design and Fabrication, VCU, Richmond, VA) controlled to deliver an equivalent impact to each animal of moderate (2.5ATM) severity. After induction of TBI, the scalp was sutured w/ 4-0 PDS and the animal was returned to a clean, warmed, home cage when ambulatory. Sham control animals underwent all procedures with the omission of the fluid percussion pulse.

Post-TBI analysis of transient loss of consciousness. Analysis of righting reflex suppression is an indicator of duration of loss of consciousness after TBI. Counting of time until return of consciousness began immediately after the percussion injury. When a conscious rat is placed on its back, it will flip to its feet or "right" itself (Floyd et al., 2002). Time to return of righting reflex after TBI was recorded and used as an indicator of loss of consciousness, a valid measure of injury severity. As shown below (Table 2, Figure 1), **the loss of consciousness for the subjects undergoing a lateral fluid percussion injury of moderate severity was greater than 2-fold longer than that for sham injured subjects**, consistent with a moderate level of brain injury. Comparison using the student's t test verified a significant difference between injury groups ($p < 0.001$).

Table 2. Righting times across injury groups with SD and SEM calculations. Data for all subjects was within 2 standard deviations of the mean for their respective groups.

	Moderate Injury	Sham
Mean Righting time (sec)	648.8	275.7
St Dev	200.8	62.7
SEM	20.6	7.1

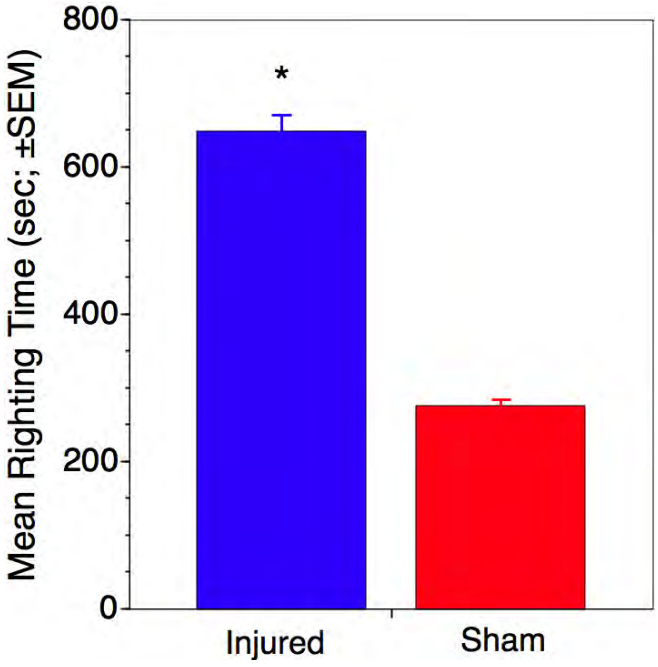

Figure 1. Shown are the mean righting times (sec; ±SEM) for all subjects undergoing either a lateral fluid percussion injury of moderate severity (Injured) or a sham injury (Sham).

Following injury, subjects were monitored closely and recovery performance recorded for 5 days. All subjects received 3 ml saline containing 5 mg/kg enrofloxacin SC, daily for 3 days. After 5 days, subjects began evaluation for the behavioral effects of oxycodone towards completion of Aims 1 and 2 as described below.

4. Tasks performed specific to Aim 1 – evaluation of acute antinociceptive effects of oxycodone and development of tolerance using a WWTW procedure

The first slated studies towards achieving this aim involved use of a warm water tail withdrawal (WWTW) procedure. Basically, the subjects were habituated to placement in a specially designed rodent restraint tube (Braintree Scientific, Braintree, MA) with their tails hanging freely out the caudal end. All subjects underwent 2 to 3 weeks of acclimation to the tubes beginning with 10 min of restraint/day and increasing to 60 min of restraint/day. Once at 60 min/day the animals continued to be habituated to the tubes every other day for 60 min until the time of injury. Habituation was suspended for 3 days following craniectomy/injury to avoid any possible effect on post injury recovery. On day 4 post-TBI, subjects were habituated for 60 min and on day 5 post-TBI, antinociception testing with oxycodone began. Thus all subjects were well acclimated to the tubes prior to antinociception testing and displayed no evidence of stress associated with the restraint. In fact subjects readily entered the tubes and were difficult to extract at the end of each habituation period.

On test days, the distal 5 cm of the tail was immersed in containers of water of different temperatures. To qualify for testing the rat had to leave his tail immersed in 40^{o} C water for 12 sec during 2 of 3 repeated exposures with 2 min between exposures. This is a non noxious stimulus and once the animals have been habituated to the restraint and to the sensation of tail immersion, almost all subjects qualify. During oxycodone testing, the subjects were exposed to noxious water temperatures of 50^{o} and 55^{o}C, consistent with our previous work (Morgan and Nicholson, 2011) and the latency to withdraw the tail was recorded. Through the use of cumulative dosing, an entire oxycodone dose effect curve was determined over approximately 2 hours. Based on procedural issues that were noted during Y1, several minor changes were made to improve reliability of data generation while also permitting retention of previously generated data. During WWTW testing in Y1 we experienced problems with tail irritation/inflammation following the initial oxycodone dose-effect curve determination. This resulted in an allodynic response for the 40^{o}C qualifying test. This painful response to a non noxious stimulus resulted in exclusion of a number of subjects from subsequent testing, particularly for the day 11 post-TBI oxycodone testing. To minimize this problem we have decreased the cutoff time for tail withdrawal from 15 to 12 sec. Additionally, following testing all subjects were placed in restraint tubes daily and tails treated with topical antibiotic/steroid ointment to minimize any inflammatory response. With these small changes in place, no subjects missed test days or were removed from the study due to tail lesions during Y2. Data generated during Y1 was reanalyzed using the 12 sec cutoff time in order to retain the data generated from as many subjects as possible. However, seven subjects (5 sham controls and 2 brain-injured) that were not able to

complete a dose-effect curve beyond the initial curve during Y1 were excluded from analysis and replaced in Y2. To summarize, the subjects were slated to undergo the following in order:

1. Habituation and training in the procedure.
2. Implantation of iPrecio preprogrammed mini pumps.
3. Sham or lateral fluid percussion injury.
4. Determination of an oxycodone dose-effect curve.
5. Repeated daily dosing every 6 hours with oxycodone or saline via the mini pump.
6. Redetermination of the oxycodone dose-effect curve after 5 days of repeated dosing (day 11 post-TBI).
7. Continue repeated dosing for an additional 5 days.
8. Determine a final oxycodone dose effect curve (day 17 post-TBI).
9. Collect brain tissue for histology and biochemistry at UAB (day 19 post-TBI).

Table 3. Shown are the numbers of subjects assigned to different injury and repeated dosing conditions across Y2 with totals for the study, Y1 and Y2, combined in parentheses.

Total number implanted with infusion pumps = 15 (47)	Total number undergoing sham injury	Repeated Dosing Assignments			Total completing testing and dosing		
		Sal	Oxy ED80	Oxy ED50	Sal	Oxy ED80	Oxy ED50
	= 6 (24)	3 (15)	3 (3)	0 (6)	3 (10)	3 (3)	0 (4)
	Total number undergoing TBI	Chronic Dosing Assignments			Total completing testing		
		Sal	Oxy ED80	Oxy ED50	Sal	Oxy ED80	Oxy ED50
	=9 (23)	6 (12)	1 (1)	0 (8)	6 (10)	1 (1)	0(5)

Pump programming and implantation. Three days prior to induction of TBI, a programmable microinfusion pump (iPrecio system, Data Sciences International, St. Paul, MN) was implanted subcutaneously under isoflurane anesthesia. The initial guidance provided by the manufacturer for surgical implantation, in our hands, was not optimal. Over the progress of several cohorts of rats combined with communication with the manufacturing company in Japan, we have evaluated different approaches and believe we have found the best location and procedure for 1) ensuring pump integrity and 2) minimizing seroma development. The rats were induced and maintained on 3% isoflurane anesthesia. They were placed in lateral recumbency and a 2-3cm surgical incision was made longitudinally through the skin just caudal to the forelimb. A pocket for the infusion pump was made using blunt dissection directed caudally. The pump was inserted in the pocket and the subcutaneous catheter tubing extending from the pump reservoir attached to a small trocar. The trocar was routed caudad and exteriorized through a small (2mm) skin incision in the lumbar region. This distal end of the tubing was disconnected from the trocar and allowed to retract under the skin. The pump itself was secured to the surrounding

fascia and musculature with two 4-0 PDS stay sutures and an additional stay suture was placed around a section of the catheter tubing. The skin incision was closed with wound clips. The pumps were programmed to run with a continuous flush of saline at a rate of 0.2 µl/hour from the time of implantation until chronic dosing began on day 5 post-TBI. Following determination of the oxycodone dose effect curve on that day, the pump reservoir was filled with saline (control group) or an oxycodone solution which provided the ED_{50} or ED_{80} dose for the 55^{o} C water stimulus determined earlier in the day in a 30 µl volume. Every 6 hours (approximately 0600,1200,1800 and 0000 hours), 30 µl of saline or oxycodone solution was released in order to mimic clinical exposure. During the intervening hours, the pump continued a low level (0.2µl/hour) flush to maintain patency of the pump tubing. The one exception to this schedule was on days 11 and 17 post-TBI when the 1200 dose was deleted from the program in order not to confound determination of the second and third dose-effect curves. Dosing stopped after the 1200 dose on day 19 post-TBI to permit collection of brain tissue between 2 and 4 hours following dosing, thus avoiding any possible spontaneous withdrawal effects in the event physical dependence had developed. Pump reservoirs were readily palpated and refilled as needed across the 19-day period.

Results to date. Figure 2 presents the baseline tail withdrawal latencies for all brain-injured (n= 17) and sham control (n=16) subjects included in the analyses at the two water temperatures regardless of subsequent dosing group assignment. As predicted, the latencies at 55^{o} were significantly shorter than the corresponding latencies at 50^{o} reflecting the greater intensity of the noxious stimulus. Baseline latencies were used to calculate %MPE = [(test latency - control latency)/ (cut-off time - control latency)] X 100% for each test point. **There was no difference in the initial response to the noxious water stimuli between subjects based on injury condition. Thus at day 5 post-TBI there is no indication of either a generalized allodynic or hyperalgesic state in subjects that had received a traumatic brain injury.** Figure 3 presents the baseline tail withdrawal latencies obtained at the onset of determination of the 3 oxycodone dose-effect curves for brain-injured and sham control subjects that received repeated saline dosing. Baseline latencies were all within normal ranges across all time points and there were no significant differences between latencies within each injury condition across the three test days. Figure 4 presents the baseline tail withdrawal latencies obtained at the onset of determination of the oxycodone dose-effect curves for brain-injured and sham control subjects that received repeated ED_{50} levels of oxycodone dosing. This testing was all performed during Y1 utilizing the longer cutoff latency and therefore the ED_{50} dose calculated under those criteria (1.2 mg/kg) was higher than any of the ED_{50} values presented from the current analysis in Table 4. As can be seen because so few subjects qualified for testing on day 11 post-TBI, the data from the second oxycodone dose-effect curve determination are not presented in the graphs. The differences noted between latencies both within and between injury conditions may reflect the relatively small number of subjects that completed testing and thus qualified for inclusion in the analysis (sham n= 4; injured n=5). Finally, Figure 5 presents the baseline latencies for the 4 subjects that were studied during Y2 that received repeated dosing with oxycodone at the ED_{80}

dose of 2.0 mg/kg every 6 hours. Insufficient subjects have currently completed this dosing condition to warrant separation of data based on injury condition. Additionally there were no overt differences in the results for the one injured subject, therefore at this point, data were combined across injury conditions. As can be seen, baseline latencies during the second dose effect curve determination were lower than those obtained during the first and third dose-effect curve determinations. While there was some concern regarding reoccurrence of Y1 problems, all tails appeared very normal, there was no nociceptive response to the 40°C water nor to moderate pressure applied to the tail tip. Finally, behaviorally, the subjects displayed a generalized agitated state. Testing was timed to occur at 1200 the time coinciding with the normal dosing time. It is possible that the subjects were actually mildly physically dependent and were exhibiting some hyperalgesia and hyperresponsiveness to stimuli associated with withdrawal. For the final oxycodone dose–effect curve, testing was performed 4 hours after the preceding dose (1000) and completed by 1200. This window was gauged to occur when any effects of the previous dose administered at 0600 had dissipated but withdrawal effects would not have begun. Alternatively these decreased baseline latencies may have been anxiety related. Basically, the subjects appeared to display anticipatory agitation due to their previous experience with the procedure. They are habituated to restraint but not the entire testing process which includes SC injections and tail immersions.

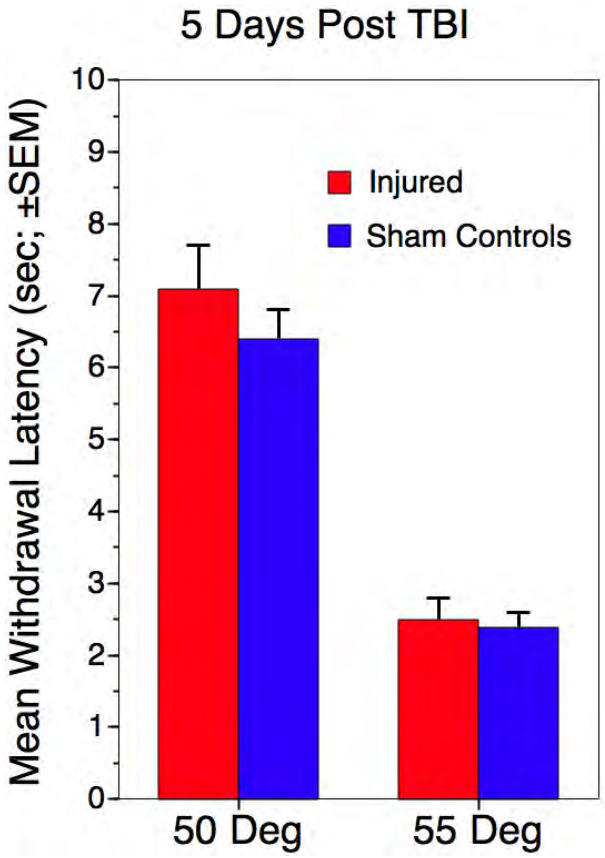

Figure 2. Shown are the baseline tail withdrawal latencies for injured and sham control subjects from 50° and 55° C water. The latencies were measured 15 min after saline administration and served as control values for determination of the % maximum possible effect for all test points following oxycodone administration. The data were collected during determination of the initial oxycodone dose-effect curve on day 5 post-TBI.

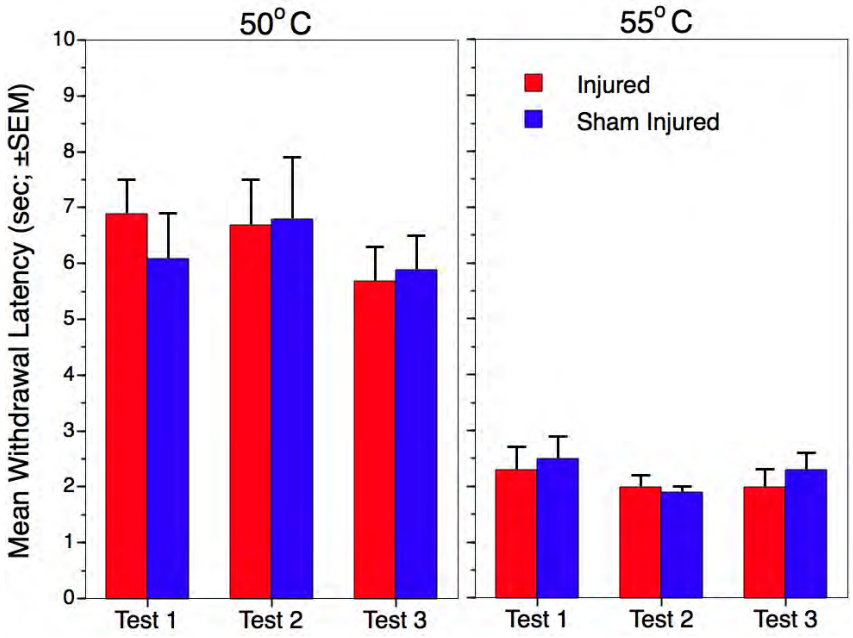

Figure 3. Shown are the baseline tail withdrawal latencies for injured and sham control subjects from 50° and 55° C water. The latencies were measured 15 min after saline administration and served as control values for determination of the % maximum possible effect for all test points. The data were collected at the onset of determination of an oxycodone dose effect curve on days 5 (Test 1) 11 (Test 2) and 17 (Test 3) post-TBI prior to and after exposure to 5 and 10 days of repeated saline, respectively.

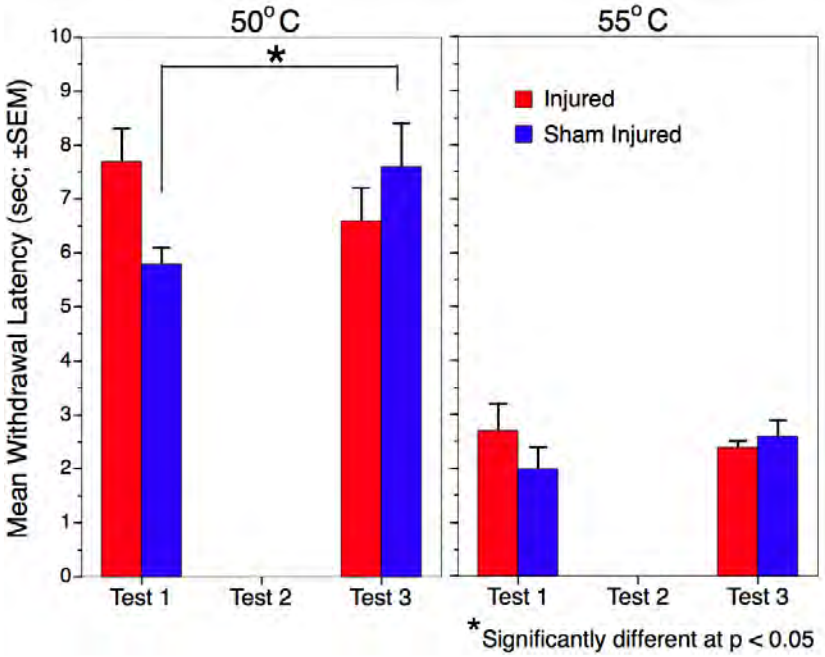

Figure 4. Shown are the baseline tail withdrawal latencies for injured and sham control subjects from 50° and 55° C water. The latencies were measured 15 min after saline administration and served as control values for determination of the % maximum possible effect for all test points. The data were collected at the onset of determination of an oxycodone dose effect curve on days 5 (Test 1) and 17 (Test 3) post-TBI prior to and after exposure to 10 days of repeated 2.0 mg/kg oxycodone, respectively.

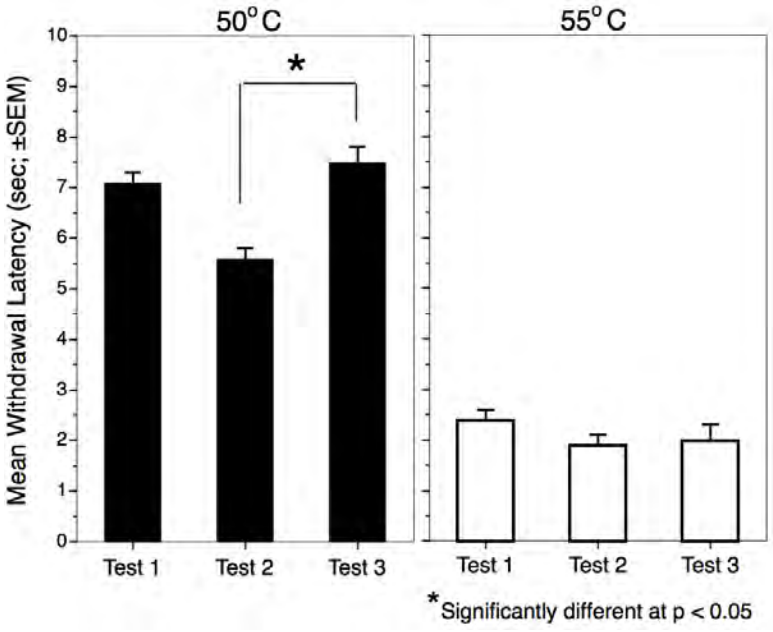

Figure 5. Shown are the baseline tail withdrawal latencies for injured and sham control subjects from 50° and 55° C water. The latencies were measured 15 min after saline administration and served as control values for determination of the % maximum possible effect for all test points. The data were collected at the onset of determination of an oxycodone dose effect curve on days 5 (Test 1) 11 (Test 2) and 17 (Test 3) post-TBI prior to and after exposure to 5 and 10 days of repeated 2.8 mg/kg oxycodone, respectively.

Figures 6 and 7 present the oxycodone dose-effect curves generated for rats before (post-TBI day 5) and after (post-TBI days 11 and 17) chronic dosing with either saline, 1.2 mg/kg oxycodone (Figure 6) or 2.0 mg/kg (Figure 7) oxycodone. As can be seen, **for all treatment/injury conditions oxycodone produced a dose-dependent antinociceptive effect at both stimulus intensities.** What did vary was the relative potency in producing these effects (Tables 4 and 5). As expected, across all treatment/injury conditions, the potency of oxycodone in producing these effects was greater at the lower intensity stimulus. ED_{50} values for oxycodone under baseline conditions (before chronic dosing) were generated using nonlinear regression analysis of the linear portion of the curves. The ED_{50} value at the 55° stimulus intensity, 1.2 mg/kg, used for chronic dosing during Y1 was based on the initial 15 sec cutoff latency and therefore was much higher than the ED_{50} values generated with the current 12 sec cutoff (Table 4). The ED_{80} value calculated using the 12 sec cutoff latency generated a dose of 2.0 mg/kg. **Following chronic dosing with oxycodone, the dose-effect curves were shifted to the right indicating that oxycodone was less potent in producing its antinociceptive effects and suggesting tolerance had developed following repeated administration of both 1.2 and 2.0 mg/kg oxycodone.** However, the dose effect curves following saline dosing were also shifted to the right to varying degrees. The differences in potency changes were more erratic at the 50°C stimulus and did not always show a greater shift for oxycodone treated versus saline treated subjects (compare injured subjects dosed with 1.2 mg/kg oxycodone to saline dosed subjects). **Overall however, the saline treated animals showed at a 2-fold or smaller decrease in oxycodone potency over testing whereas the oxycodone treated animals showed a 2-fold or greater decrease in potency.** There are several possible causes of the shifts seen in the saline animals. An initial concern was that we were continuing to experience imperceptible levels of damage to the tails and hyperalgesia. However, as stated earlier there

was no supporting evidence for this. Another possibility is that neuroadaptation associated with tolerance development occurred even after the limited exposure to oxycodone. Acute tolerance has been reported in humans and nonhuman subjects after a single dose of an opioid and is often referred to as acute opioid-induced hyperalgesia (Chu et al., 2008, Lee et al., 2011). Another possible explanation relates to experience with the procedure. Subjectively, the rats appeared more agitated and overtly responsive to injections during tests two and three suggesting that as familiarity with the procedure increased, anticipatory anxiety also increased resulting in a general hyper-responsiveness to stimuli. When comparing across injury condition, the oxycodone dose effect curves were remarkably similar as shown in Figure 4. **Overall, based on the data generated to date, there does not appear to be a difference in the acute antinociceptive effects of oxycodone or in the development of tolerance to those effects between sham and brain-injured subjects in a model of acute spinally-mediated nociception.**

Table 4. Shown are the ED_{50} values (mg/kg) for oxycodone's WWTW antinociceptive effects across both injury conditions prior to and after repeated dosing with oxycodone (1.2 mg/kg) or saline.

	Test 1		Test 3 after Repeated Saline		Test 1		Test 3 after Repeated Oxycodone	
Temp (^{o}C)	50^{o}	55^{o}	50^{o}	55^{o}	50^{o}	55^{o}	50^{o}	55^{o}
Injured Subjects	0.23	0.65	0.45*	1.10*	0.57	0.60	0.40	3.46*
95% CL	0.15-0.35	0.44-0.97	0.26-0.67	0.76-1.56	0.41-0.79	0.46-0.77	0.01-10.58	2.27-5.28
Sham Controls	0.24	0.73	0.51	0.80	0.30	0.70	0.92*	3.68*
95% CL	0.16-0.36	0.53-0.99	0.35-0.74	0.43-1.47	0.21-0.44	0.54-0.89	0.38-2.24	0.22-60.66

*significantly different from Test 1 value at $p < 0.05$.

Table 5. Shown are the ED_{50} values (mg/kg) for oxycodone's WWTW antinociceptive effects prior to and after repeated dosing with oxycodone (2.0 mg/kg).

	Test 1		Test 3 after Repeated Oxycodone	
Temp (^{0}C)	50^{o}	55^{o}	50^{o}	55^{o}
All subjects	0.29	1.25	0.97	2.87*
95% CL	0.16-0.53	1.06-1.47	0.89-1.05	2.21-3.73

*significantly different from Test 1 value at $p < 0.05$.

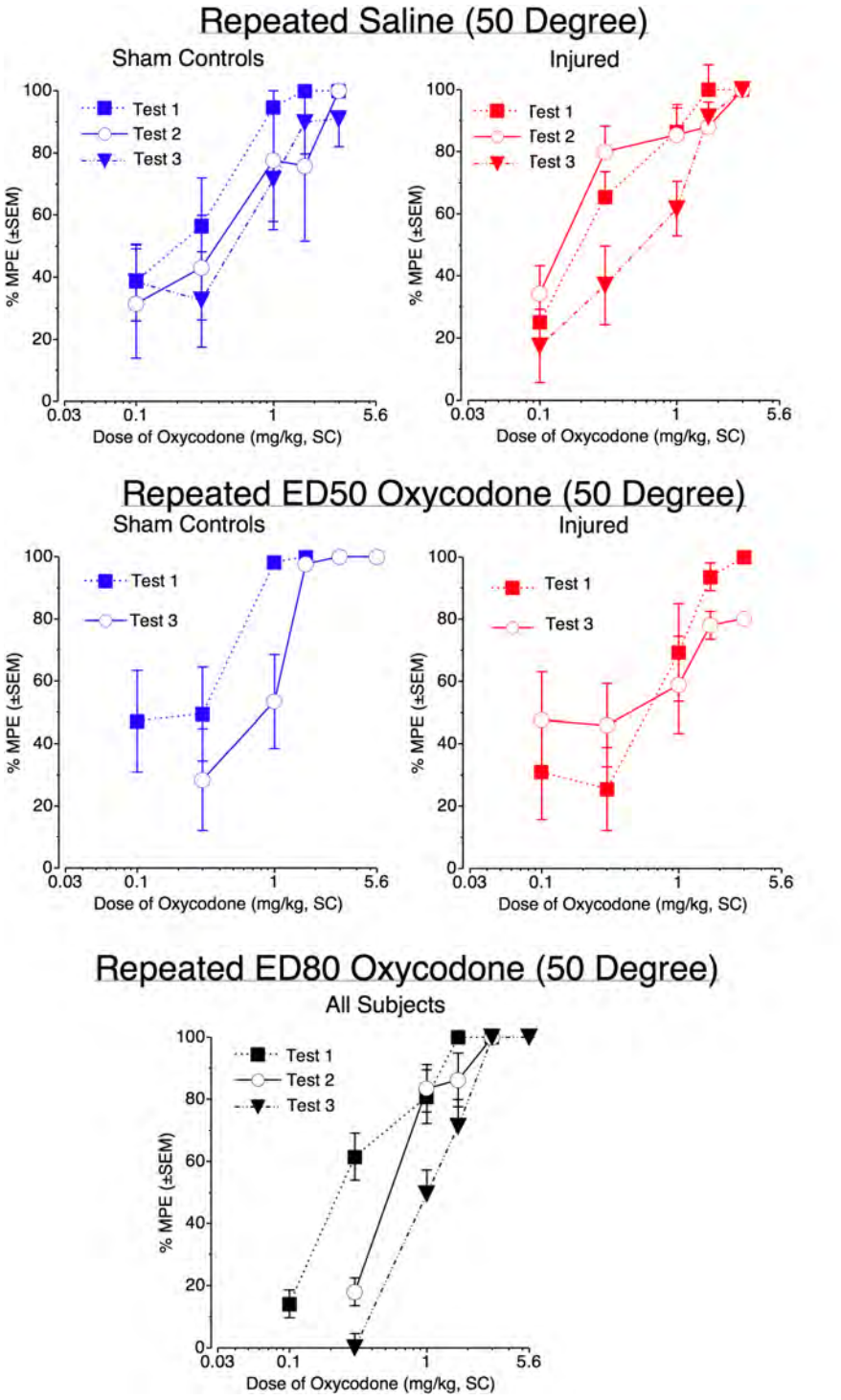

Figure 6. Shown is the percent maximum possible effect for oxycodone antinociceptive effects at the lower stimulus intensity following repeated dosing with saline (top panel), 1.2 mg/kg oxycodone (middle panel) or 2.0 mg/kg oxycodone (bottom panel).

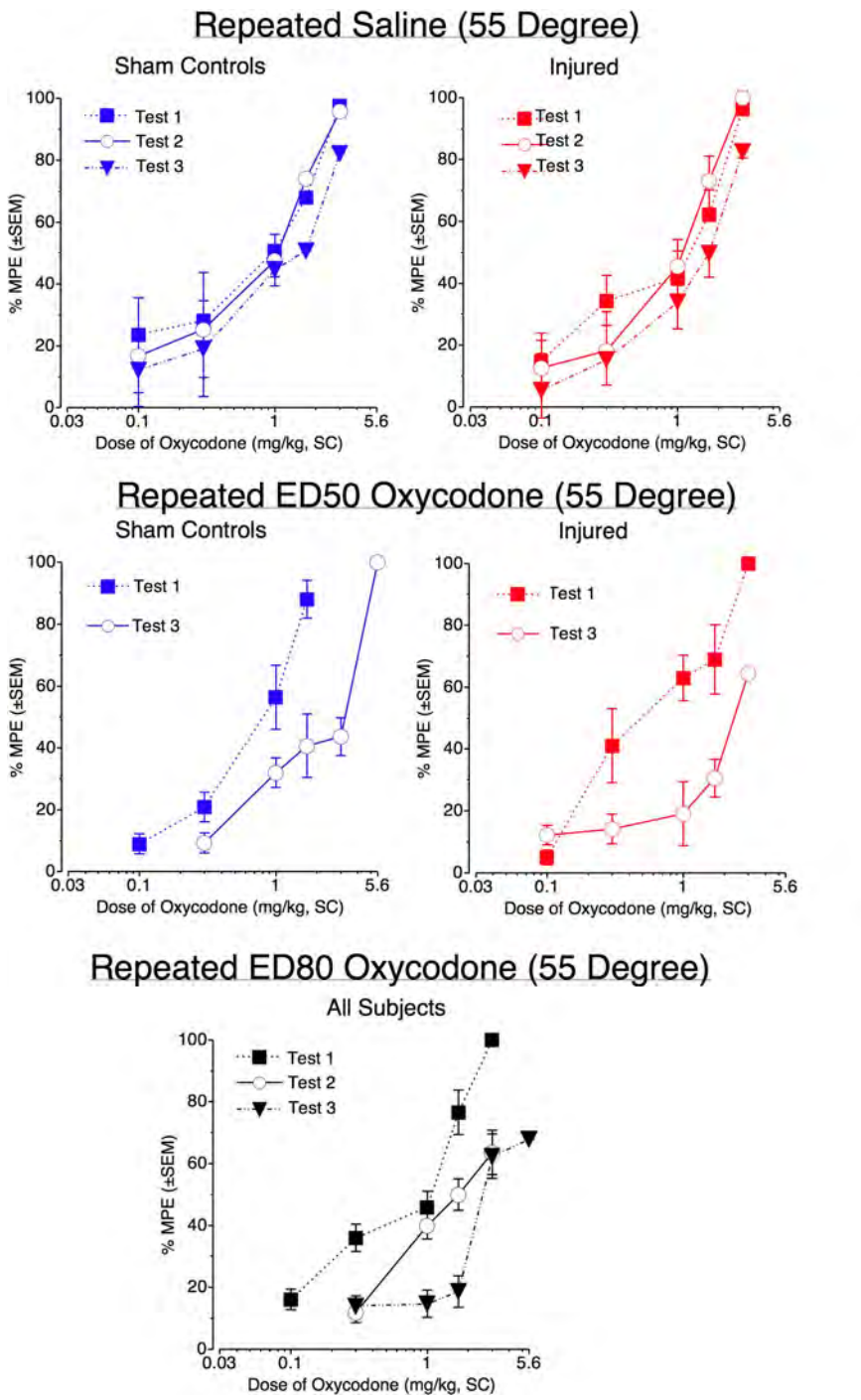

Figure 7. Shown is the percent maximum possible effect for oxycodone antinociceptive effects at the higher stimulus intensity following repeated dosing with saline (top panel), 1.2 mg/kg oxycodone (middle panel) or 2.0 mg/kg oxycodone (bottom panel).

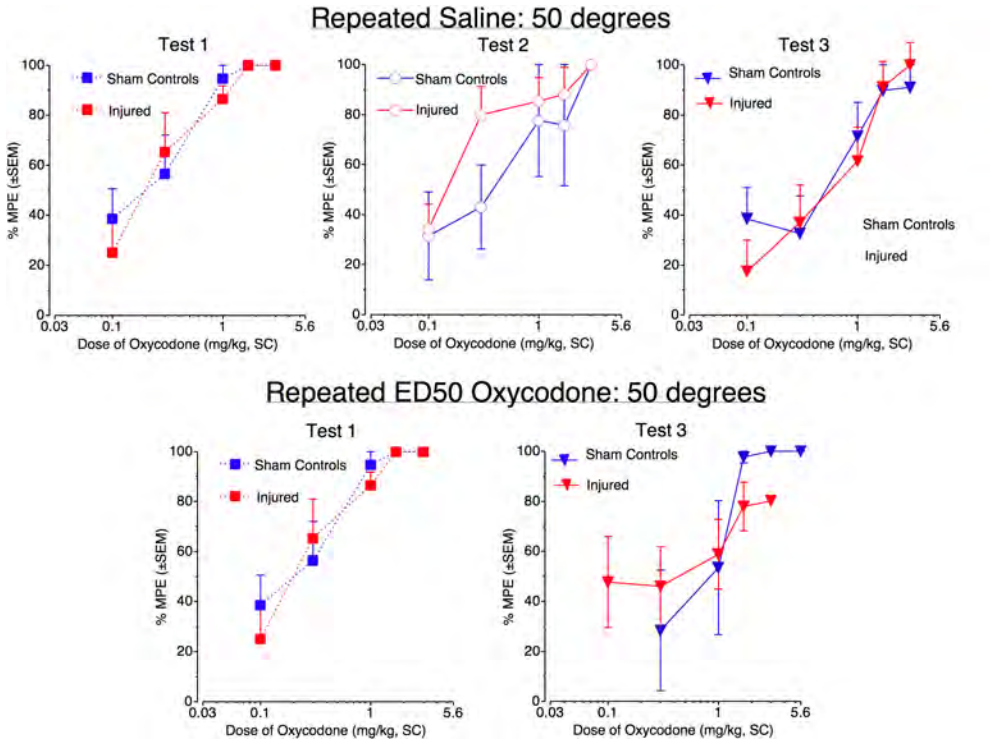

Figure 8. Shown is the percent maximum possible effect for oxycodone antinociceptive effects at the lower stimulus intensity across injury conditions following repeated dosing with saline (top panel) or 1.2 mg/kg oxycodone (bottom panel).

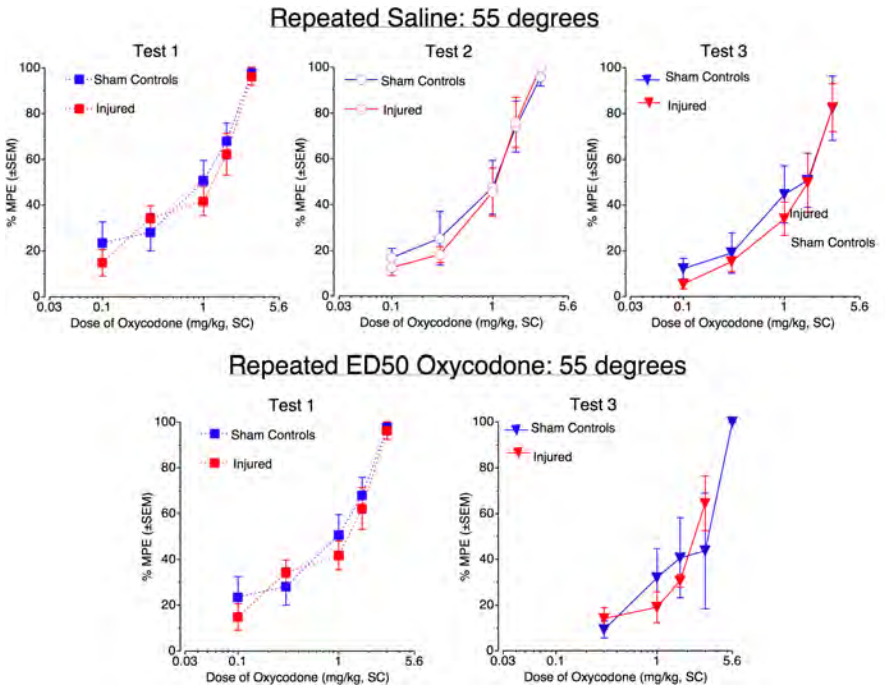

Figure 9. Shown is the percent maximum possible effect for oxycodone antinociceptive effects at the higher stimulus intensity across injury conditions following repeated dosing with saline (top panel) or 1.2 mg/kg oxycodone (bottom panel).

Tissue sample collection. 48 hours after the final dose effect curve determination approximately 50% of the subjects were deeply anesthetized with an overdose of pentobarbital (>150 mg/kg) and their brains rapidly removed. Brains were flash frozen in an isopentane bath cooled in a dry ice/methanol slurry and stored at -80° C until shipment to UAB. The remaining 50% similarly received an overdose of pentobarbital (>150 mg/kg) and underwent perfusion with 4% formalin.

Table 6. Details of samples prepared for analysis at UAB during Y2 as well as the total collected over Y1 and Y2 combined:

Injury Condition	Chronic Treatment	Preparation	Number Y2	Total Number
Sham injured	Saline	Frozen	3	7
Sham injured	Saline	Perfused	3	8
Sham injured	Oxycodone ED50	Frozen	0	2
Sham injured	Oxycodone ED50	Perfused	0	4
Sham injured	Oxycodone ED80	Frozen	1	1
Sham injured	Oxycodone ED80	Perfused	2	2
Injured	Saline	Frozen	3	7
Injured	Saline	Perfused	4	6
Injured	Oxycodone ED50	Frozen	0	6
Injured	Oxycodone ED50	Perfused	0	4
Injured	Oxycodone ED80	Frozen	1	1
Injured	Oxycodone ED80	Perfused	0	0

5. Tasks performed specific to Aim 1 – evaluation of acute antinociceptive effects of oxycodone and development of tolerance using hotplate response

Studies examining the effect of brain injury on opioid tolerance development using a supraspinally mediated model of acute pain were initiated in Y2. Unlike the WWTW procedure, hotplate nociception/antinociception does not require any habituation to restraint for the procedure. Indeed, as will be discussed below, familiarity with the procedure may contribute to changes observed in baseline responses. All subjects underwent 1 week of acclimation to the laboratory, personnel and handling prior to implantation of iPrecio programmable pumps. Pump implantation was as described for WWTW procedure. On day 5 post-TBI baseline oxycodone antinociceptive effects were determined. Testing began with a SC saline injection (1 ml/kg) followed 15 min later by placement onto a heated metal plate (iiTC Life Science, Woodland Hills, CA) set at 52.5^{o} C. The subjects were confined to the plate by a bottomless clear acrylic enclosure and latency to lick a paw or exhibit escape behavior measured. A cutoff of 40 sec was imposed to avoid tissue damage. All subjects had paws examined repeatedly following testing. No subjects displayed any inflammation or heat associated lesions across the testing days. As with WWTW procedure, pumps were initially filled with saline delivered at 0.2 μl/hour until day 5 post-TBI. Immediately following the initial oxycodone dose-effect curve determination, the saline was extracted from the pump reservoir and it was refilled with either saline or the concentration of oxycodone that would

deliver 2.8 mg/kg (determined ED_{80} value) in 30 µl. The oxycodone dose or saline was delivered every 6 hours until 1200 hours on day 19 post-TBI, excluding the 1200 h dose on days 11 and 17 post-TBI, at which time subjects were euthanized and brains collected. To summarize, the subjects were slated to undergo the following in order:

1. Habituation to the laboratory and handling.
2. Implantation of iPrecio preprogrammed mini pumps.
3. Sham or lateral fluid percussion injury.
4. Determination of an oxycodone dose-effect curve.
5. Repeated daily dosing with oxycodone or saline via the mini pump.
6. Redetermination of the oxycodone dose-effect curve after 5 days of repeated dosing (day 11 post-TBI).
7. Continue repeated dosing for an additional 5 days.
8. Determine a final oxycodone dose effect curve (day 17 post-TBI).
9. Collect brain tissue for histology and biochemistry at UAB (day 19 post-TBI).

Table 7. Shown are the numbers of subjects assigned to different injury and chronic dosing conditions during Y2.

<table>
<tr><td rowspan="6">Total number implanted with infusion pumps
= 35</td><td rowspan="2">Total number undergoing sham injury</td><td colspan="2">Chronic Dosing Assignments</td><td colspan="2">Total completing testing and dosing</td></tr>
<tr><td>Sal</td><td>Oxy ED80</td><td>Sal</td><td>Oxy ED80</td></tr>
<tr><td>= 15</td><td>10</td><td>4</td><td>10</td><td>4</td></tr>
<tr><td rowspan="2">Total number undergoing TBI</td><td colspan="2">Chronic Dosing Assignments</td><td colspan="2">Total completing testing</td></tr>
<tr><td>Sal</td><td>Oxy ED80</td><td>Sal</td><td>Oxy ED80</td></tr>
<tr><td>= 20</td><td>10</td><td>3</td><td>10</td><td>2</td></tr>
</table>

At this time, because only 6 total animals have completed testing following repeated oxycodone dosing (2 injured and 4 sham controls), their data were collapsed across injury condition.

Results to Date:

A comparison of the baseline latencies for all sham controls and brain-injured subjects (Figure 10) shows that **there was no significant difference based on injury condition in responding to the nocicieptive stimulus 5 days post-TBI.** This was consistent with testing in the WWTW procedure. When we extend that comparison out across the three dose-effect curve determinations, we observe a trend in the sham controls to show shorter latencies to respond to the stimulus, however this effect was not significant. A similar and significant effect was noted in the subjects that were assigned to receive the ED_{80} dose of oxycodone for repeated dosing

(Figure 11). This decreasing latency to respond was greatest between the first and second dose-effect curve determinations but then leveled out or even increased when measured for the third dose-effect curve determination. The possible explanations for the change in baseline include: 1) Initial unfamiliarity with the apparatus, testing procedure and potential escape strategies resulting in longer latency to display overt nociception (licking paw, distress behaviors); 2) coincident with the increasing familiarity with the procedure was anticipation of the exposure to the noxious stimulus resulting in an elevation in anxiety and hyper-responsiveness to the stimulus; 3) imperceptible tissue damage resulting in actual peripheral hyperalgesia; and 4) for the ED_{80} group, generalized hyperalgesia associated with drug withdrawal as discussed for WWTW. Based on the observed behaviors of the animals, the frequent examinations of the paws and that testing was performed within a temporal window that should have avoided withdrawal effects, the most probable explanations are a combination of factors 1 and 2.

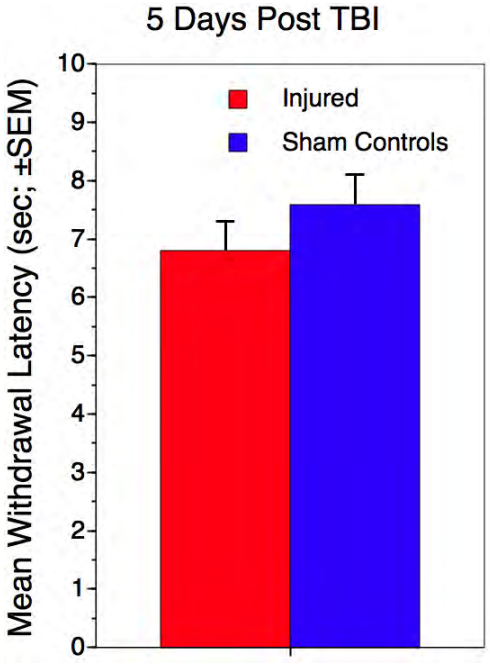

Figure 10. Baseline withdrawal latencies from 52.5°C hotplate surface determined 5 days following moderate TBI (Injured; n=10) or sham control injury (Sham; n=10).

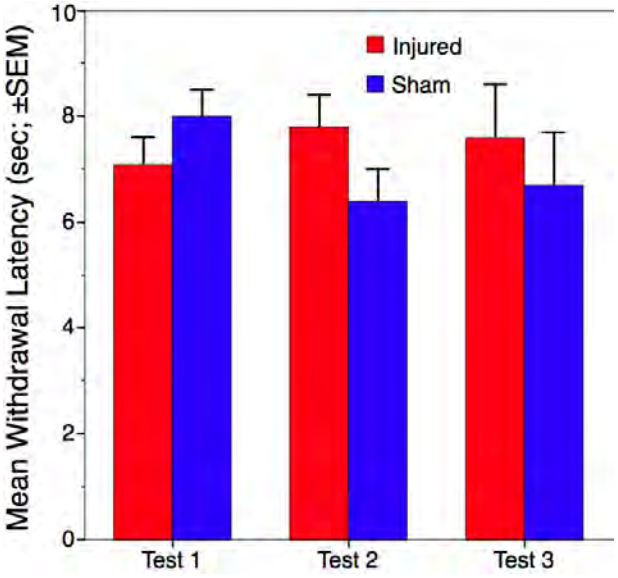

Figure 11. Baseline withdrawal latencies across the three test days (days 5, 11 and 17 post-TBI) for sham controls and moderately injured subjects receiving repeated saline administration.

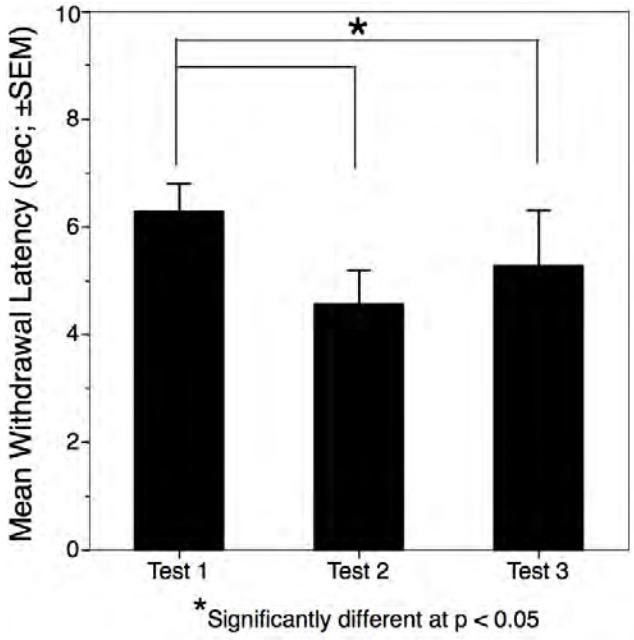

Figure 12. Baseline withdrawal latencies across the three test days (days 5, 11 and 17 post-TBI) for all subjects receiving repeated 2.8 mg/kg oxycodone administration. Baseline withdrawal latencies decreased significantly following the initial round of testing.

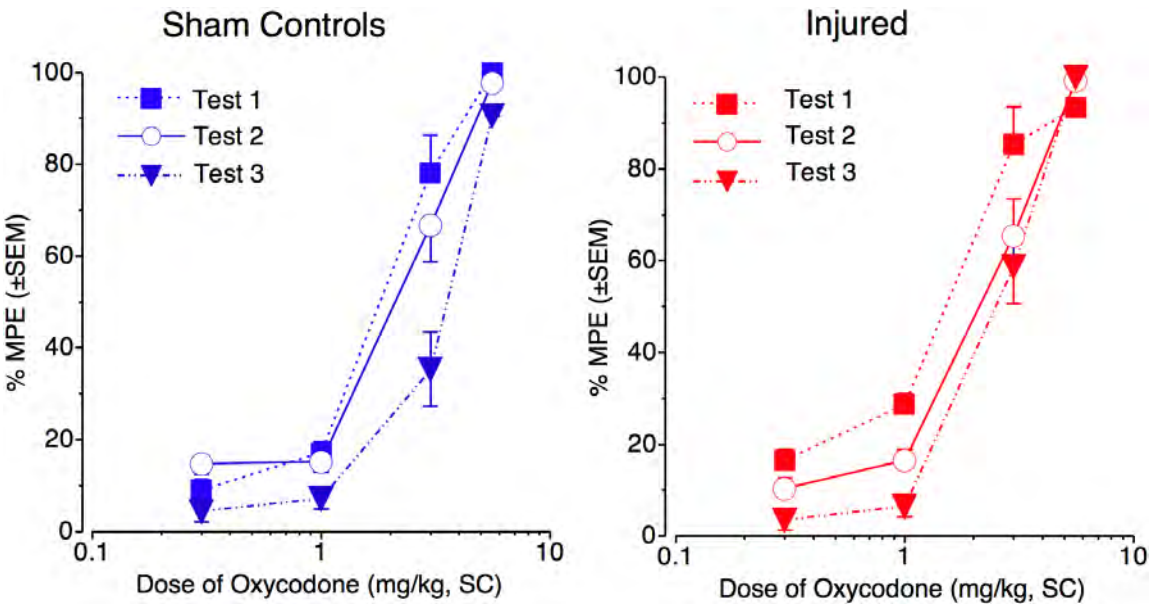

Figure 13. Shown is the percent maximum possible effect for oxycodone antinociceptive effects in the hot plate procedure following repeated dosing with saline compared within injury condition across the three test days.

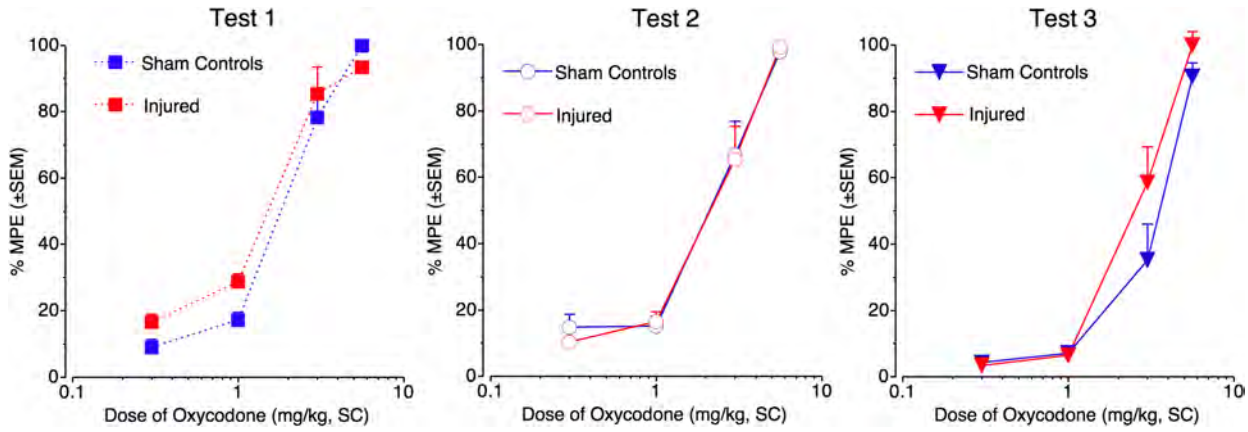

Figure 14. Shown is the percent maximum possible effect for oxycodone antinociceptive effects in the hot plate procedure following repeated dosing with saline compared between brain-injured and sham control subjects across the three test days.

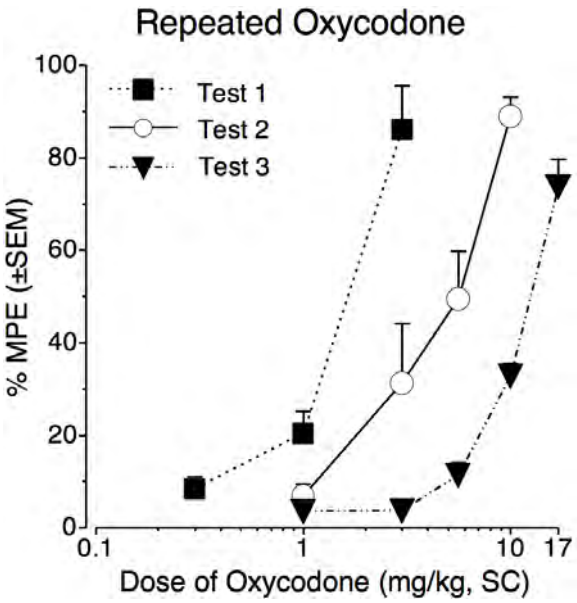

Figure 15. Shown is the percent maximum possible effect for oxycodone antinociceptive effects in the hot plate procedure following repeated dosing with 2.8 mg/kg oxycodone across the three test days. Data for brain-injured and sham control subjects are combined.

For all subjects, oxycodone produced a dose-dependent increase in antinociception. When comparing the effects of oxycodone across test days in subjects that received repeated saline over the 2-week period, similar to the WWTW procedure we see a modest (30%), but significant decrease in potency of oxycodone (Figure 13 and Table 8). This effect was the same regardless of injury condition. The virtually identical results for the two groups is further illustrated by Figure 14 where the curves for the two injury conditions are almost superimposed. Only preliminary testing has been completed (n=2 for injured and n=4 for sham controls) for the development of tolerance following repeated administration of the ED_{80} dose of oxycodone. As can be seen in Figure 15 and Table 8, there was a progressive rightward shift in the dose-effect curves over the course of the study suggesting tolerance development in these subjects. The change in potency after 5 days of repeated oxycodone exposure was similar to that seen in the

saline treated subjects (40% decrease). By the third and final dose-effect curve determination the decrease in potency was over 7-fold, highly suggestive of development of tolerance to oxycodone but because of the limited statistical power provided by the currently smaller number of subjects, this decrease in potency was not significant. At this time, insufficient brain-injured subjects have been tested to permit comparison of the effects across our injury conditions. What this does show however is that our model and dosing conditions are consistent across cohorts and able to produce readily measurable tolerance development.

Table 8. Shown are the ED_{50} values (mg/kg) for oxycodone's antinociceptive effects in a hotplate procedure prior to and after repeated dosing with oxycodone (2.8 mg/kg) or saline. The data compared are those from the first and third oxycodone dose-effect curve determinations.

	Baseline Day 5 Post-TBI	Post Repeated Saline Day 17 Post-TBI	Post Repeated Oxycodone Day 17 Post-TBI
Brain-injured (n=10)	1.55	2.34	
95% CL	1.27-1.88	2.01-2.72	
Sham Controls (n=10)	1.86	2.96	
95% CL	1.61-2.14	2.39-3.67	
ED_{80} subjects (n=6)	1.60		11.91
95% CL	1.64-1.99		10.77-13.18

Tissue sample collection. 48 hours after the final dose effect curve determination approximately 50% of the subjects were deeply anesthetized with an overdose of pentobarbital (>150 mg/kg) and their brains rapidly removed. Brains were flash frozen in an isopentane bath cooled in a dry ice/methanol slurry and stored at -80° C until shipment to UAB. The remaining 50% similarly received an overdose of pentobarbital (>150 mg/kg) and underwent perfusion with 4% formalin.

Table 9. Details of samples prepared for analysis at UAB during Y2:

Injury Condition	Chronic Treatment	Preparation	Number Y2
Sham injured	Saline	Frozen	5
Sham injured	Saline	Perfused	5
Sham injured	Oxycodone ED80	Frozen	2
Sham injured	Oxycodone ED80	Perfused	2
Injured	Saline	Frozen	5
Injured	Saline	Perfused	5
Injured	Oxycodone ED80	Frozen	1
Injured	Oxycodone ED80	Perfused	1

6. Tasks performed specific to Aim 2 assessing the rewarding properties of oxycodone in injured versus sham injured subjects

The first slated studies towards completing this aim assessed acquisition of oxycodone self-administration. Upon arrival animals were acclimated to handling and the laboratory environment. Five days prior to injury, animals underwent surgical implantation of a chronic indwelling venous catheter under isoflurane anesthesia with morphine pretreatment. A surgical incision was made longitudinally through the skin above the jugular area. The underlying fascia was bluntly dissected and the external jugular vein isolated and ligated with 5-0 silk suture. A small cut was made into the vein using an iris scissors and the catheter introduced into the vein to a level near but not into the right atrium. The vein encircling the catheter was then tied with 5-0 silk suture. A second suture was used to anchor the catheter to surrounding fascia. The rat was then placed ventral side down on the surgical table and a 2 cm incision was made 1 cm lateral from mid-scapula. A second 0.3 cm incision was then made mid-scapula. The distal end of the catheter was passed subcutaneously from the ventrum (vein cannulation area) to the larger dorsal incision and attached to the cannula/connector. The cannula/connector was then inserted subcutaneously through the larger incision while the upper post portion of the connector/cannula exits through the smaller mid-scapular incision. All incisions were sprayed lightly with a gentamicin/ betamethasone valerate topical antibiotic and the larger dorsal incision and ventral neck incision closed with michel wound clips. Catheters were flushed daily with amoxicillin/sublactam (20/10 mg/kg) in a saline/glycerol/heparin solution to enhance catheter longevity. Periodic infusion of 7.5mg/kg ketamine IV was used to verify catheter patency by presence of immediate onset of sedation.

As shown in Table 10 below, 60 subjects have completed testing in the self-administration acquisition procedure. Five days following TBI or sham injury described above, subjects began daily self-administration testing conducted in standard operant chambers housed within isolated and ventilated enclosures (Med Associates). Each chamber was equipped with two response levers with a white stimulus light above each lever, a 5-watt house light in the rear wall and an adjustable Sonalert (Model ENV-223AM, Med Associates) in the upper left wall. During each session, infusion tubing, protected by a stainless steel spring tether (Plastics One), was connected to the back-mounted cannula pedestal. Infusions were delivered via a peristaltic pump located outside each chamber. Schedule parameters were controlled by MED-PC IV software (Med Associates) running on a PC compatible computer. Rats were brought to the laboratory daily (7 days/week) and allowed to acclimate for 15-30 min before being connected to the infusion tether and placed in the chamber for the 2-hour acquisition session. During the session, a single response, fixed ratio (FR) 1, on the right lever resulted in the delivery of a 0.1-ml, 3-sec infusion of one of the three oxycodone doses as shown in Table 10. Responding on the left lever had no scheduled consequence but was recorded as a measure of behavioral activity in the chamber. Criteria for acquisition was three consecutive days of receiving > 15

infusions and responses on the active lever > responses on the inactive lever. Subjects were permitted up to 21 sessions to achieve criteria.

In Y2, 34 subjects began acquisition testing and 25 completed testing, the remaining 9 (3 sham, 2 injured) lost catheter patency prior to 21 days. This higher than predicted rate of loss reflects results with a single cohort that had an unexpectedly high rate of catheter loss (30%) within the first two weeks of testing for unknown reasons. Overall, we have now completed acquisition testing in 60 (30 injured, 30 sham) across the originally proposed 3 oxycodone doses. Of these subjects, 10 (5 injured, 5 sham) failed to acquire self-administration behavior. As expected, availability of 0.03 mg/kg/infusion dose resulted in the highest percent acquisition in both the injured (100%) and sham control subjects (90%). The lowest dose, 0.003 mg/kg/infusion, resulted in acquisition levels (50% of injured and 60% of sham controls) relatively consistent with our predicted range of 40-50%. Availability of the intermediate dose of oxycodone was associated with a significant difference in acquisition levels between the injured rats and sham controls with the injured animals demonstrating 100% acquisition versus 60% for the controls (Figure 16A). What did not show considerable difference between the infusion doses was the mean rate of acquisition as shown in Figure 16B. Analysis of the daily cumulative acquisition percent for the sham and injured subjects as shown in Figures 17 and 18 provides additional insight into the patterns for acquisition and reaffirms the difference in behavior between the sham controls and the brain-injured subjects at the intermediate dose. **This difference suggests that the brain-injured subjects are more sensitive to the reinforcing/rewarding effects of oxycodone.**

Table 10. Shown are the numbers of subjects assigned to different injury and dosing conditions across Y2 with total numbers for Y1 and Y2 combined in parentheses.

Number catheterized	Number undergoing sham injury	Number entering acquisition	Number completing acquisition	Oxycodone Dose (mg/kg/infusion)		
				0.003	0.01	0.03
= 41 (96)	= 24 (51)	=22 (39)	=16 (30)	6 (10)	6 (10)	4 (10)
	Number undergoing TBI	Total entering acquisition procedure	Total completing acquisition assessment	Oxycodone Dose (mg/kg/infusion)		
				0.003	0.003	0.003
	=17 (55)	= 12 (35)	= 9 (30)	4 (10)	2 (10)	3 (10)

Following acquisition, the rats were allowed to continue self-administering the acquisition dose until performance was stable. Stability was defined as 3 days in which the number of infusions did not differ from the mean for the 3 days by more than 20% with no increasing or decreasing trends. The mean number of infusions across those three days is shown in Figure 19. Dose-dependent differences are present between the two injury groups with the sham control animals maintaining their highest levels of responding at the intermediate dose of oxycodone. In addition, there was an inverted U-shaped dose-effect curve relating dose per infusion to infusion rate for the sham control rats. This type of dose-effect relationship is typical of drugs effective

as positive reinforcers (Young and Herling, 1986). For the brain-injured subjects there was a dose-dependent increase in infusions across the three oxycodone doses and the total number of infusions at the highest oxycodone dose was significantly greater than the amount self-administered by the sham control subjects. Comparing the shapes of the dose-effects curves, it appears that we have only tested the ascending limb of the inverted "U"-shaped curve for the brain-injured subjects. Taken alone, these data might suggest that the reinforcing effects of oxycodone were less potent in the brain-injured subjects, however the acquisition data suggest the opposite. **Alternatively, the data could suggest that the brain-injured subjects are <u>less</u> sensitive to the <u>use-limiting</u> effects of the oxycodone (sedation, dysphoria, etc) and will self-administer a greater total dose of oxycodone compared to sham control subjects.** In humans this propensity to take more drug and higher total doses of drug has been posited to increase risk for escalating drug use and addiction (de Wit and Phillips, 2012). Based on these results, an additional cohort of subjects has been added to each injury condition to test a higher (0.056 mg/kg/infusion) dose of oxycodone. Testing in these subjects was just initiated at the end of July 2013 after receiving VCU IACUC and ACURO approval. Additionally, separate groups of injured and sham control subjects are being evaluated for gross behavioral effects at different doses of oxycodone to determine the relative potency of oxycodone for producing locomotor activity effects in an open field in the two injury groups.

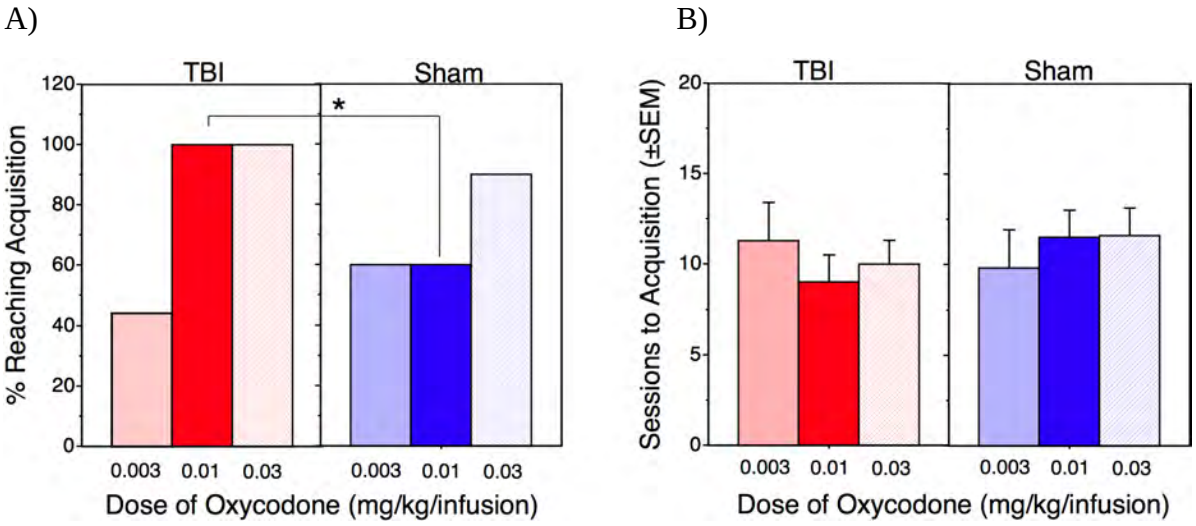

Figure 16. Panel A) Shown are the percent of brain-injured (TBI) and sham control subjects acquiring self-administration behavior across the different oxycodone doses. (n=30 for sham controls, n=30 for brain-injured subjects). * significantly different from sham controls at $p < 0.05$. Panel B) Shown are the mean number of days to achieve self-administration acquisition criteria for sham and brain-injured (TBI) subjects across the three oxycodone doses.

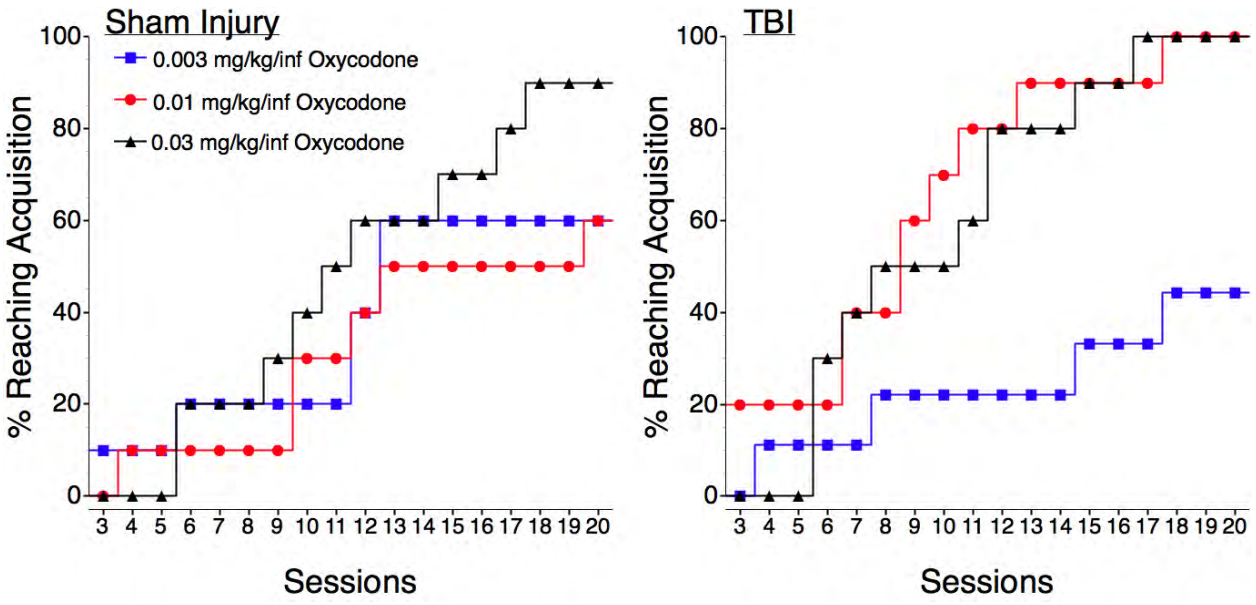

Figure 17. Shown are the cumulative percentages of subjects acquiring self-administration behavior across sessions for sham controls (left panel) and brain-injured (right panel) rats across the three oxycodone doses.

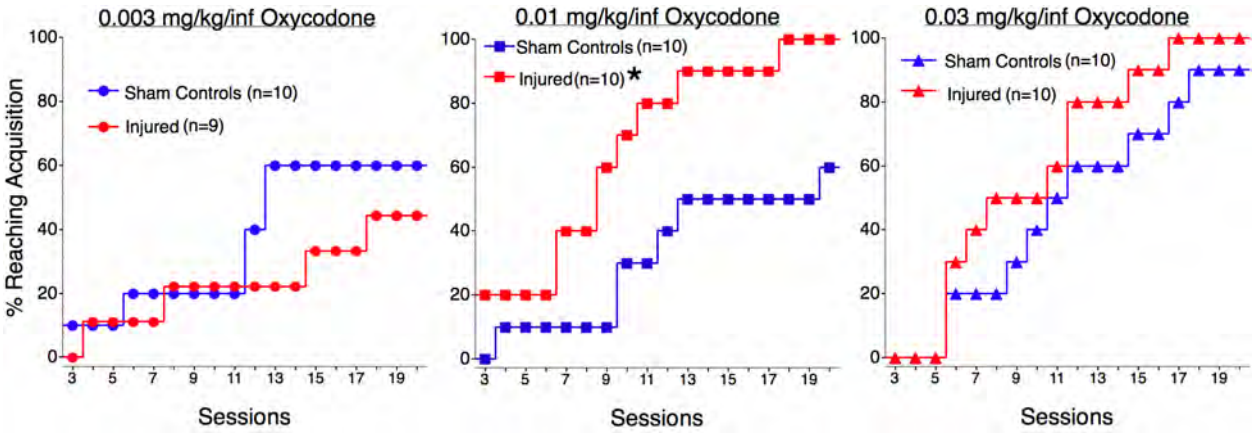

Figure 18. Shown are the cumulative percentages of subjects acquiring self-administration behavior across sessions for sham control and brain-injured rats for each of the three oxycodone doses. * significantly different from sham controls at $p < 0.05$.

The primary dependent measures collected during acquisition testing included the number of days to meet the acquisition criterion, the percentage of rats per group meeting the criteria, and the number of infusions received following stabilization of responding. However, data regarding a number of other parameters were also routinely collected. For example, responding on the inactive lever was recorded. This responding serves as a measure of general activity within the chamber. As expected, responding was significantly lower on the inactive relative to the active lever across all oxycodone doses in both the injured rats and sham controls that

acquired self-administration behavior (Figure 20). This demonstrates that responding for oxycodone was serving as a positive reinforcer of behavior and that responding on the active lever was not simply by chance but was goal-directed. Previous differences noted in Y1 between the injured subjects and sham controls in the level of responding on the inactive lever dissipated as the number of subjects increased supporting that basal activity levels were not different between the groups.

A final measure that was analyzed was responding which occurred on the active lever during the 2-min timeout that was imposed following each infusion. During the 3-sec infusion of drug solution, the houselight was extinguished and the stimulus light over the lever was illuminated. When the infusion was complete, the stimulus light was also extinguished and the operant chamber remained unlit for the 2-min timeout period. Responding was recorded but did not result in delivery of drug or any light response. At the end of the timeout, the houselight was once again illuminated and responding on the active lever resulted in drug presentation. As shown in Figure 21, the brain-injured subjects elicited a significantly greater number of responses during the timeout period than the sham controls. Timeout responding has been viewed as a potential measure of drug seeking however others perceive it as a measure of impulse control – can the subject wait for the timeout to end and the active period begin before responding again. Either explanation could account for what was seen in our study. Therefore we have added another group of brain-injured and sham controls to examine responding for food reward – a nondrug reinforcer of behavior. These 24 subjects underwent injury and initiated testing at the end of July 2013 after VCU IACUC and ACURO approval was received. Results from this group will permit determination of whether the greater timeout responding was reflective of drug seeking or due to generalized dysfunction of impulse control.

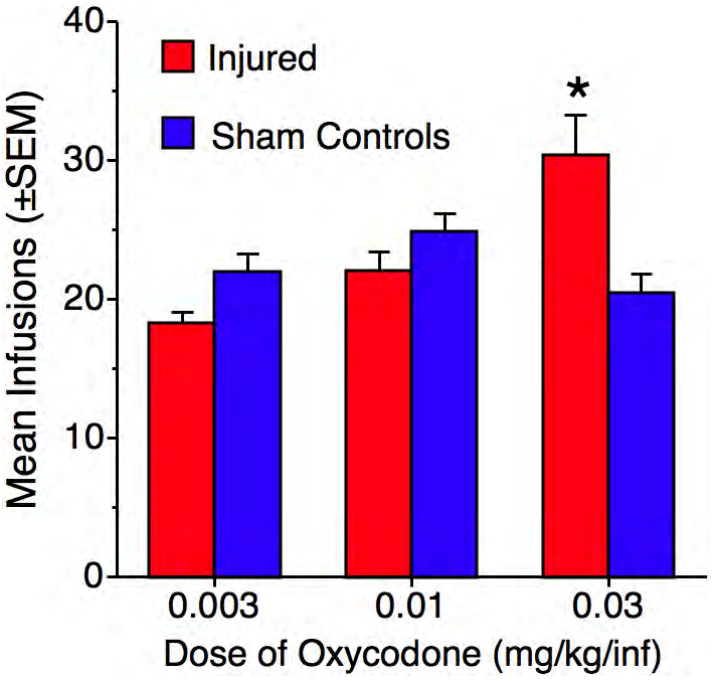

Figure 19. Shown are the mean number of oxycodone infusions self-administered across three days of stable responding comparing levels for sham controls to brain-injured rats across the three acquisition doses. * significantly different from sham controls at $p < 0.05$.

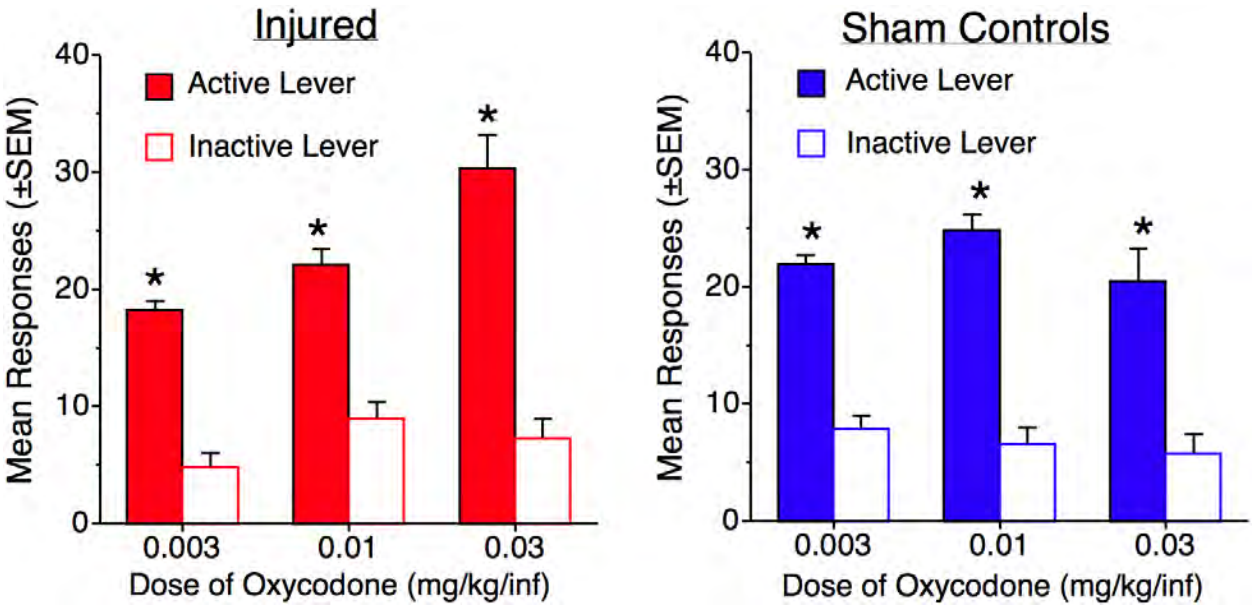

Figure 20. Shown are the mean number of lever responses emitted on the active (closed bars) and inactive (open bars) levers during three days of stable responding for oxycodone self administration for sham controls (left panel) and brain-injured (right panel) rats. * significantly different from inactive lever responding at $p < 0.05$.

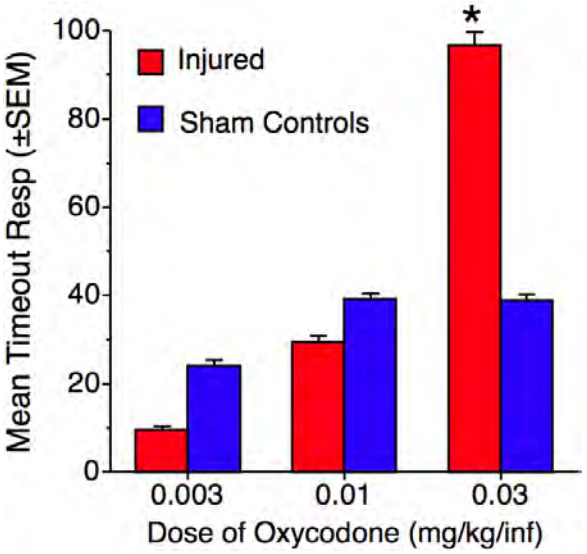

Figure 21. Shown are the mean number of lever responses emitted on the active lever during the timeout period over the three days of stable responding for oxycodone self administration for sham controls and brain-injured rats. * significantly different from sham controls at $p < 0.05$.

Tissue sample collection. Minimally 72 hours after the final self-administration session, approximately 50% of the subjects were deeply anesthetized with an overdose of pentobarbital (>150 mg/kg) and their brains rapidly removed. Brains were flash frozen in an isopentane bath cooled in a dry ice/methanol slurry and stored at -80° C until shipment to UAB. The remaining

50% similarly received an overdose of pentobarbital (>150 mg/kg) and underwent perfusion with 4% formalin.

Table 11. Details of tissue samples collected for analysis at UAB in Y2 (Y1 and Y2 combined in parentheses).

Injury Condition	Preparation	Number
Sham injured	Frozen	8 (15)
Sham injured	Perfused	8 (15)
Injured	Frozen	4 (15)
Injured	Perfused	5 (15)

7. Tasks performed specific to Aim 2 assessing relapse-like behavior following oxycodone self-administration in injured versus sham injured subjects

Fourteen subjects have completed testing of reinstatement behavior in our goal to assess the effect of moderate brain injury on relapse-like behavior. Subjects were prepared with indwelling intravenous catheters and underwent sham or moderate injury procedures as described for the acquisition study. Based on findings in the acquisition study, the dose of 0.03 mg/kg/infusion was selected for use in the reinstatement studies. Therefore all subjects assigned to reinstatement assessment began training in self-administration of 0.03 mg/kg/infusion of oxycodone on day 5 post-TBI as outlined in Table 12. Unlike the acquisition procedure, any subject not demonstrating minimally 10 infusions/session by day 3 of training was primed (received an infusion through investigator initiated responding) and also had the lever baited with peanut butter to promote responding. Of the 17 rats that entered reinstatement training/testing, 1 subject failed to acquire the behavior within the allotted 14-day period. As soon as subjects met the same acquisition criteria and reached stable levels of responding as described above for acquisition, they entered extinction training. During extinction sessions, subjects were placed in the operant chambers and connected to the tethers. The behavioral session was initiated as signaled by illumination of the houselight and presentation of the response levers. However, responding on either lever had no scheduled consequence – no drug infusion, no extinguishing of the houselight, no stimulus light presentation. Subjects were given up to 14 days of extinction training to meet extinction criteria. To be considered to have extinguished, the number of total responses emitted by each subject had to be less than 50% of their baseline oxycodone administration level and responding over three extinction days had to be within 25% of the mean of the three days with no trends. Two subjects failed to meet extinction criteria within the 14-day time window. The remaining 14 subjects met extinction criteria, and for most, particularly the brain-injured subjects, responding was well below the 50% of control level by the time stability was achieved. Once extinction criteria were met, subjects underwent prime- and cue–induced resinstatement of responding. All subjects underwent both reinstatement conditions with minimally 3 and up to 7 days of renewed extinction training in between the sessions (until extinction responding was once again stable). Prime- and cue-induced sessions were counterbalanced across subjects within each injury

condition to compensate for any order effects. During cue-induced reinstatement sessions, the initial response on the active lever resulted in presentation of the drug-associated cues: houselight extinction, stimulus light presentation and 2-min timeout period followed by return to extinction session conditions for all subsequent responses. During prime-induced reinstatement sessions, 1 mg/kg oxycodone was administered SC 15 min prior to session onset otherwise the prime reinstatement behavioral session was identical to the extinction session.

Table 12. Shown are the numbers of subjects assigned to different injury and dosing conditions across Y1

Number catheterized	Number undergoing sham injury	Number entering Reinstatement procedure	Number completing Reinstatement procedure
21	=9	=8	=7
	Number undergoing TBI	Number entering Reinstatement procedure	Number completing Reinstatement procedure
	=12	=9	=7

Figures 22 and 23 present reinstatement responding for sham controls and injured subjects. Unlike the acquisition procedure, responding for oxycodone under baseline conditions was not higher for the brain-injured subjects compared to the sham controls. This is likely due to the more rapid self-administration training and more limited total time of oxycodone exposure during reinstatement training compared with acquisition. Whereas subjects in the reinstatement procedure had acquired the behavior and reached stability within 14 days, acquisition subjects were permitted up to 21 days (mean time was approximately 10 days) with additional access permitted for reaching stability. This meant many subjects in the acquisition study continued self-administering oxycodone for up to 30 days total. During that time, responding in the brain-injured subjects continued to increase modestly resulting in the higher final intake for brain-injured versus sham controls. It required an average of 10.3 and 8.7 days for injured and sham control subjects to reach extinction criteria, respectively. While this suggests the injured subjects took longer to extinguish, at this point in data collection, the difference is not significant. Once behavior was extinguished however, injured subjects exhibited lower baseline responding during extinction sessions, and this difference was significant for responding prior to cue-induced reinstatement. Baseline extinction responding was calculated based on the mean responses emitted during the 3 extinction training sessions preceding the prime- or cue-induced reinstatement sessions. As can be seen (Figure 22), even the single cue presentation resulted in a significant increase in responding by both sham controls and brain-injured subjects. This is consistent with numerous studies examining relapse-like behavior in both preclinical and clinical studies (Shaham et al., 2003; Crombag et al., 2008; Smith and Aston-Jones, 2012). Indeed, the effect of cues has proven to be a significant cause of relapse in human substance

abuse and has been shown to maintain high levels of responding following reinstatement for psychostimulants and opioids in preclinical studies. While it did result in modestly increased levels of responding, the oxycodone prime (Figure 23) did not result in a significant increase in levels of responding relative to baseline. This may be due to inadequate statistical power at this point in the study but may also reflect the dose of oxycodone used for reinstatement. The dose selected was based on responses in the antinociception testing and in the literature. The 1 mg/kg dose in antinociception testing did not appear to cause overt sedation/motor suppression, however approximately half of the first ten subjects tested were obviously intoxicated at this dose and exhibited few if any responses during the behavioral session. Therefore, in the subsequent 4 subjects, we performed an additional reinstatement trial with 0.3 mg/kg dose of oxycodone prior to the session and are seeing far more behavior during reinstatement. The data presented in this report reflect testing at the 1 mg/kg dose. We will complete reinstatement testing in the remaining 6 subjects using both doses of oxycodone in order to present the most accurate assessment of relapse-like behavior in our two populations.

Given the level of variability between subjects in terms of baseline extinction responding, cue- and prime-induced responding data were also assessed as a percent of the preceding extinction sessions for both cue and prime-induced reinstatement (right hand panels in Figures 22 and 23). **Whereas there appeared to be a greater reinstatement in the sham controls relative to injured subjects based on number of responses, when evaluated in the context of baseline responding, we see there is no difference in reinstatement behavior between the two injury conditions based on the data to date.**

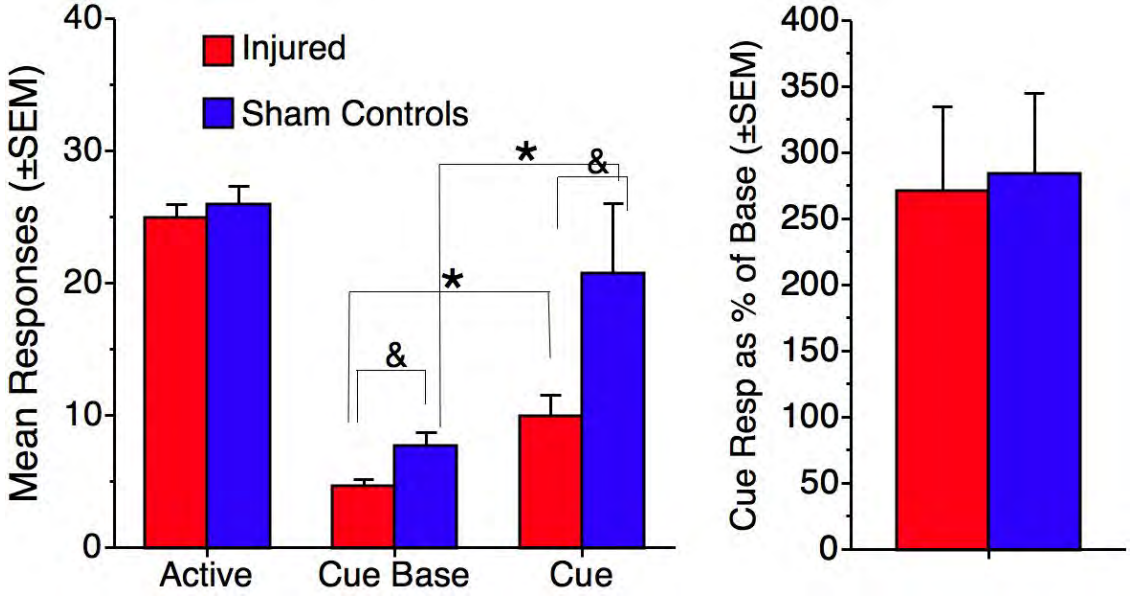

Figure 22. Shown in the lefthand graph are the mean number of responses on the active lever during oxycodone reinforced sessions (Active), following extinction training (Cue Base) and during cue-induced reinstatement for sham controls (n=7) and brain-injured (n=7) rats. The righthand graph presents responding during the cue-induced session expressed as a percentage of each subjects Cue Base control levels.

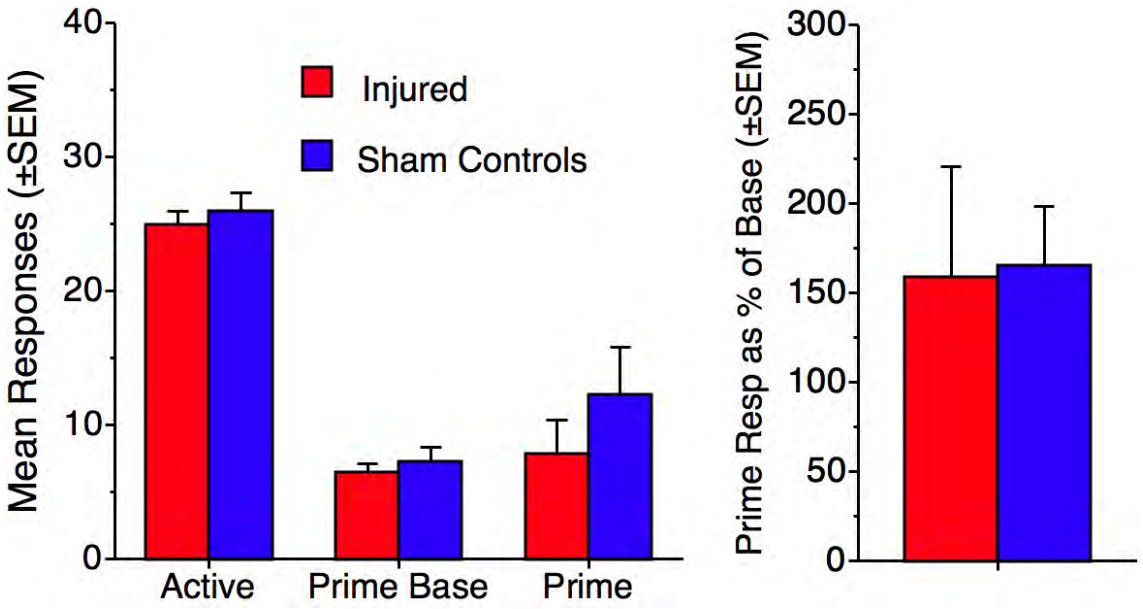

Figure 23 Shown in the lefthand graph are the mean number of responses on the active lever during oxycodone reinforced sessions (Active), following extinction training (Prime Base) and during prime-induced reinstatement for sham controls (n=7) and brain-injured (n=7) rats. The righthand graph presents responding during the cue-induced session expressed as a percentage of each subjects Prime Base control levels.

Tissue sample collection. Minimally 72 hours after the final extinction session, approximately 50% of the subjects were deeply anesthetized with an overdose of pentobarbital (>150 mg/kg) and their brains rapidly removed. Brains were flash frozen in an isopentane bath cooled in a dry ice/methanol slurry and stored at -80° C until shipment to UAB. The remaining 50% similarly received an overdose of pentobarbital (>150 mg/kg) and underwent perfusion with 4% formalin.

Table 13. Details of tissue samples prepared and sent to UAB

Injury Condition	Preparation	Number
Sham injured	Frozen	4
Sham injured	Perfused	3
Injured	Frozen	3
Injured	Perfused	4

8. Summary:

At this time, we have completed testing for acquisition of oxycodone self-administration across three doses and demonstrated interesting and important differences between the injured subjects and the sham controls in acquisition behavior. We also have completed a significant portion of our investigation into the effect of brain injury on relapse like behavior. At this point, there appears to be no difference based on injury condition in the propensity to relapse to

oxycodone self-administration once behavior is extinguished, but results are still preliminary. We have completed all analgesia testing of oxycodone in subjects administered repeated doses of saline in our two pain models. All testing has been optimized such that only 1 subject entering behavioral testing had to be removed from the study (pump failure). Analysis of the saline treated subjects shows a trend towards a decreasing potency of oxycodone over testing, but this decrease was less than the potency decrease seen in subjects administered repeated doses of oxycodone across the same dosing period. This study evaluating the propensity for tolerance development between the two injury conditions is ongoing. All subjects added to the SOW in the final month of Y2 (24 self administration subjects and 20 food maintained subjects) underwent injury and began behavioral assessment at the end of July 2013. All behavioral testing for these animals will be complete by end of August 2013. Animals slated for testing in July of Y3 have also been purchased, acclimated and undergone injury and behavioral assessment either in July or August 2013 so we remain on track for completion as scheduled in the SOW.

Key findings to date:

- **There was no difference in baseline nociception (pain threshold) between the sham controls and the brain-injured subjects in either the spinally or supra-spinally mediated measures of acute pain. This suggests that moderate brain injury does not result in a generalized allodynia or hyperalgesia.**

- **There was no difference in the response to oxycodone between sham controls and brain-injured subjects when treated repeatedly with saline. There was some indication that a higher level of tolerance may develop in brain-injured subjects when dosed repeatedly with oxycodone but data are too preliminary to draw a final conclusion.**

- **The intermediate dose of oxycodone resulted in a significantly higher percentage of brain-injured subjects than sham controls acquiring the self-administration behavior suggesting the injured subjects are more sensitive to the reinforcing (rewarding) effects of oxycodone.**

- **The brain-injured subjects appeared to be less sensitive to the use-limiting effects of oxycodone self-administering the greatest number of infusions at the highest dose, and at a significantly greater level compared to the sham control subjects.**

- **The brain-injured subjects demonstrated a significantly greater level of perseverative responding for oxycodone as shown by higher responding during timeout periods suggesting an increase in impulsive behavior and/or drug seeking relative to sham controls.**

- **Preliminary results showed that self-administration behavior extinguished slightly more slowly in brain-injured subjects however once extinguished, responding was significantly lower in the brain-injured subjects. At this time there is no apparent difference in reinstatement of responding between the two injury conditions following both cue- and prime-induced reinstatement.**

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